PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07H 21/04, C07K 16/00, C12N 1/14, 1/20, 5/00, 9/90, 15/00, C12P 21/06, C12Q 1/68

A1

(11) International Publication Number: WO 99/07727

(43) International Publication Date: 18 February 1999 (18.02.99)

(21) International Application Number: PCT/US98/16593

(22) International Filing Date: 10 August 1998 (10.08.98)

(30) Priority Data: 60/055,586 12 August 1997 (12.08.97) US

(71) Applicant: MASSACHUSETTS INSTITUTE OF TECHNOLOGY [US/US]; 77 Massachusetts Avenue, Cambridge, MA 02139 (US).

(72) Inventors: KAISER, Chris, A.; 153 C Mount Auburn Street, Cambridge, MA 02138 (US). FRAND, Alison, R.; Apartment 402, 143 Albany Street, Cambridge, MA 02139 (US).

(74) Agent: ELBING, Karen, L.; Clark & Elbing LLP, 176 Federal Street, Boston, MA 02110-02214 (US).

(81) Designated States: CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: EUKARYOTIC DISULFIDE BOND-FORMING PROTEINS AND RELATED MOLECULES AND METHODS

(57) Abstract

Disclosed herein are expression systems that make use of Erol to enhance disulfide bond formation and thereby to increase the yield of properly folded recombinant proteins. Also disclosed herein are recombinant Erol polypeptides, nucleic acids, vectors, and cells for expressing such polypeptides.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

EUKARYOTIC DISULFIDE BOND-FORMING PROTEINS AND RELATED MOLECULES AND METHODS

Statement as to Federally Sponsored Research

This invention was made in part with support from the Government through NIH Grant No. ROI GM46941. The Government has certain rights in the invention.

Background of the Invention

This invention relates to novel eukaryotic disulfide bond-forming proteins and uses thereof, particularly for increasing yields of recombinant proteins produced in in vivo or in vitro expression systems.

Many commercially produced proteins are cell surface or extracellular proteins that contain cysteine residues capable of forming disulfide bonds in the oxidizing environment of the endoplasmic reticulum (ER). For these proteins to assume their proper active folded conformation, the cysteine residues must be linked by disulfide bonds in a correct pairwise arrangement, a process that is catalyzed by cellular enzymes. One such enzyme involved in both the formation and rearrangement of disulfide bonds in eukaryotic cells is the abundant ER protein disulfide-isomerase (PDI). Protein production strategies to maximize the yield of disulfide bond-containing proteins have made use of PDI, either by overproducing PDI in cells expressing a protein of interest or by mixing a denatured protein substrate with purified PDI in in vitro refolding systems. In either case, even the use of excess PDI has generally resulted in only a modest increase in the yield of properly folded protein, and has sometimes catalyzed instead the formation of insoluble protein

aggregates.

Summary of the Invention

In general, the invention features a method of increasing disulfide bond formation in a protein (for example, a secreted protein) involving: (a) denaturing the protein; and (b) allowing renaturation of the protein in the presence of an Ero1 polypeptide (formerly known as a Sec81 polypeptide). In a preferred embodiment of this method, the Ero1 polypeptide is combined with a protein disulfide-isomerase. In another embodiment, the Ero1 polypeptide is derived from a yeast.

In another aspect, the invention features a method of increasing disulfide bond formation in a protein (for example, a secreted protein), involving expressing the protein in a host cell that also expresses an isolated nucleic acid that encodes an Ero1 polypeptide. In a preferred embodiment of this method, the host cell further expresses a nucleic acid encoding a protein disulfide-isomerase. In another embodiment, the Ero1 polypeptide is derived from a yeast.

In another aspect, the invention features a substantially pure preparation of an Ero1 polypeptide, which may be derived from a yeast or from a mammal (for example, a human). In preferred embodiments, the Ero1 polypeptide includes an amino acid sequence which is at least 27%, preferably at least 50%, more preferably at least 60%, and most preferably at least 80% identical to the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 29, or alternatively which exhibits at least 50%, preferably, at least 70%, more preferably at least 80%, and most preferably at least 90% sequence identity to SEQ ID NOS: 3, 4, 5, 6, 7, 8, 9, or 10, or any combination thereof.

The invention also features isolated nucleic acid encoding an Ero1

polypeptide. This isolated nucleic acid is preferably at least 27%, more preferably 50%, and most preferably at least 75% identical to the nucleotide sequence of SEQ ID NO: 1 or SEQ ID NO: 28, or encodes an Ero1 polypeptide which either includes an amino acid sequence that is at least 27%, preferably at least 50%, more preferably at least 60%, and most preferably at least 80% identical to the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 29, or exhibits at least 50%, preferably at least 70%, more preferably at least 80%, and most preferably at least 90% sequence identity to SEQ ID NOS: 3, 4, 5, 6, 7, 8, 9, or 10 or any combination thereof. This nucleic acid may include the sequence of SEQ ID NO: 1 or SEQ ID NO: 28, or, in a preferred embodiment, may complement an Ero1 mutation in yeast (for example, *S. cerevisiae*).

The isolated nucleic acid encoding an Ero1 polypeptide may be included in a vector, such as a vector that is capable of directing the expression of the protein encoded by the nucleic acid in a vector-containing cell. The isolated nucleic acid in the vector can be operatively linked to a promoter, for example, a promoter that is capable of overexpressing the Ero1 polypeptide, or that is capable of expressing Ero1 in a conditional manner. The isolated nucleic acid encoding an Ero1 polypeptide, or a vector including this nucleic acid, may be contained in a cell, such as a bacterial, mammalian, or yeast cell.

Also included in the invention is a method of producing a recombinant Ero1 polypeptide, and an Ero1 polypeptide produced by this method. This method involves (a) providing a cell transformed with isolated nucleic acid that encodes an Ero1 polypeptide and is positioned for expression in the cell under conditions for expressing the isolated nucleic acid, and (b) expressing the recombinant Ero1 polypeptide.

A substantially pure antibody, such as a monoclonal or polyclonal antibody, that specifically recognizes and binds an Ero1 polypeptide is also

included in the invention. Preferably, the Ero1 polypeptide is derived from a yeast.

The invention also features a method of detecting a gene, or a portion of a gene, that is found in a mammalian cell (for example, a human cell) and that has sequence identity to the Ero1 sequence of Fig. 1A (SEQ ID NO: 1) or to the Ero1 sequence of Fig. 10 (SEQ ID NO: 28). In this method, isolated nucleic acid encoding the Ero1 polypeptide, a portion of such nucleic acid greater than about 15 residues in length, or a degenerate oligonucleotide corresponding to one or more Ero1 conserved domains (for example, SEQ ID NOS: 3, 4, 5, 6, 7, 8, 9, or 10), is contacted with a preparation of nucleic acid from the mammalian (for example, human) cell under hybridization conditions that provide detection of nucleic acid sequences having about 50% or greater nucleic acid sequence identity. If desired, this method may also include a step of testing the gene, or portion thereof, for the ability to functionally complement a yeast *Ero1* mutant (e.g., a *S. cerevisiae Ero1* mutant).

Another method included in the invention is a method of isolating a gene, or a portion of a gene, that is found in a mammalian cell (for example, a human cell) and has at least 50%, preferably at least 70%, more preferably at least 80%, and most preferably at least 90% sequence identity to a sequence encoding SEQ ID NOS: 3, 4, 5, 6, 7, 8, 9, or 10. This method involves (a) amplifying by PCR the mammalian gene, or portion thereof, using oligonucleotide primers having regions of complementarity to opposite nucleic acid strands in a region of the nucleotide sequence of Fig. 1A (SEQ ID NO: 1) or of Fig. 10 (SEQ ID NO: 28), and (b) isolating the mammalian gene, or portion thereof. This method can also include a step of testing the gene, or portion thereof, for the ability to functionally complement a yeast *Ero1* mutant (e.g., a *S. cerevisiae Ero1* mutant).

As used herein, by an "Ero1" polypeptide is meant a polypeptide, formerly known as a Sec81 polypeptide, derived from a eukaryote that promotes disulfide bond formation and whose function may be substituted by an exogenous oxidant, such as diamide (for example, under conditions as described herein).

By "substantially pure" is meant a preparation which is at least 60% by weight (dry weight) the compound of interest, e.g., an Ero1 polypeptide. Preferably the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99% by weight the compound of interest. Purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

By "isolated nucleic acid" is meant nucleic acid that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally-occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant nucleic acid which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences. It also includes a recombinant nucleic acid which is part of a hybrid gene encoding additional polypeptide sequence.

By a "substantially identical" polypeptide sequence is meant an amino acid sequence which differs from a reference sequence only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative substitutions, deletions, or insertions

located at positions of the amino acid sequence which do not destroy the function of the polypeptide (assayed, e.g., as described herein).

Preferably, such a sequence is at least 75%, more preferably at least 85%, and most preferably at least 95% identical at the amino acid level to the sequence used for comparison.

Sequence identity is typically measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705, or BLAST software available from the National Library of Medicine). Examples of useful software include the programs, Pileup and PrettyBox. Such software matches similar sequences by assigning degrees of homology to various substitutions, deletions, substitutions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

By a "substantially identical" nucleic acid is meant a nucleic acid sequence which encodes a polypeptide differing only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative substitutions, deletions, or insertions located at positions of the amino acid sequence which do not destroy the function of the polypeptide (assayed, e.g., as described herein). Preferably, the encoded sequence is at least 75%, more preferably at least 85%, and most preferably at least 95% identical at the amino acid level to the sequence of comparison. If nucleic acid sequences are compared, a "substantially identical" nucleic acid sequence is one which is at least 85%, more preferably at least 90%, and most

preferably at least 95% identical to the sequence of comparison. The length of nucleic acid sequence comparison will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably at least 100 nucleotides. Again, identity is typically measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705).

By "positioned for expression" is meant that the nucleic acid molecule is positioned adjacent to a sequence which directs transcription and translation of the nucleic acid molecule.

By "purified antibody" is meant antibody which is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, antibody.

By "specifically binds" is meant an antibody which recognizes and binds an Ero1 polypeptide but which does not substantially recognize and bind other molecules in a sample (e.g., a biological sample) which naturally includes the Ero1 polypeptide. An antibody which "specifically binds" such a polypeptide is sufficient to detect protein product in such a biological sample using one or more of the standard immunological techniques available to those in the art (for example, Western blotting or immunoprecipitation).

By "complementation" is meant an improvement of a genetic defect or mutation.

The present invention provides an important advance in this field of technology. For example, the identification of Ero1 provides a simple and

inexpensive means to increase the production of commercially important disulfide bond-containing proteins. Because Ero1 may be recombinantly expressed in combination with a commercial protein of interest or may be used as an isolated and purified reagent, the present invention enables the enhancement of disulfide bond formation during in vivo commercial protein production or at subsequent in vitro purification steps, or both. Moreover, to further maximize disulfide bond formation, Ero1 proteins may be used in conjunction with other disulfide bond-forming enzymes, such as PDI proteins. Proper formation of disulfide bonds results in the production of batches of recombinant proteins exhibiting higher yields of properly folded products; this maximizes protein activity and minimizes the presence of species capable of triggering immunological side effects.

Other features and advantages of the invention will be apparent from the following detailed description thereof, and from the claims.

Brief Description of the Drawings

Figure 1A is the nucleic acid sequence of the coding strand of the *S. cerevisiae* Ero1 DNA (SEQ ID NO: 1) shown in the 5' to 3' direction.

Figure 1B is the amino acid sequence of the *S. cerevisiae* Ero1 polypeptide (SEQ ID NO: 2) shown in the amino-terminal to carboxy-terminal direction.

Figures 2A, 2B, and 2C are autoradiographs showing that the *ero1-1* mutation causes a defect in ER to Golgi transport for a subset of proteins, as compared to the *sec12* mutation, which unilaterally blocks ER to Golgi transport. Wild-type (CKY10), *ero1-1* (CKY559) and *sec12* (CKY39) strains were grown at 24°C, and then shifted to 37°C. Upon shifting to the

higher temperature, the strains were pulse labeled with [^{35}S] methionine and cysteine for 7 minutes, followed by a chase with an excess of unlabeled methionine and cysteine. The effect of DTT was tested by addition of 5 mM DTT to the cultures 10 minutes before labeling. Protein immunoprecipitated from labeled extracts was resolved by SDS-PAGE. Shown in **Fig. 2A** are the results of carboxypeptidase Y (CPY) immunoprecipitation at 10, 30, and 60 minutes after initiation of the chase. The p1 (ER) and m (vacuole) forms of the CPY protein are as indicated. **Fig. 2B** shows the results of Gas1p immunoprecipitation at 10, 30, and 60 minutes after initiation of the chase. A *sec6* mutant (CKY560) was additionally used to show prevention of degradation of mature Gas1p at the cell surface. The precursor (ER) and mature (Golgi) forms of Gas1p are as indicated. In **Fig. 2C**, cells carrying pNV31 (a pTPI1-SUC2 fusion) were labeled for 10 minutes and then converted to spheroplasts for the detection of invertase. Invertase was then immunoprecipitated from spheroplasts (int) and supernatant (ex) fractions. The positions of the core-glycosylated ER form and the mature Golgi and secreted forms of invertase are as indicated.

Figures 3A and 3B are autoradiographs demonstrating that the *ero1-1* mutation causes a defect in disulfide bond formation in CPY. **Fig. 3A** shows nonreducing and reducing gels demonstrating that in an *ero1-1* mutant incubated at 37°C, the p1 form of CPY comigrates with the reduced p1 form of CPY. **Fig. 3B** shows that in an *ero1-1* mutant incubated at 37°C, the p1 form of CPY has free thiols that react with acetamido-4'-maleimidylstilbene-2,2'-disulfonate (AMS).

Figure 4A is an autoradiograph of pulse-chase labeled Ero1p-myc showing that the 96 kD Ero1p-myc protein expressed at high levels from a high copy number plasmid (lane 3) is reduced to 81 kD following treatment with

EndoH (lane 4), indicating that Ero1p is an N-linked glycoprotein.

Figure 4B is an autoradiograph of pulse-chase labeled Ero1p-myc and invertase immunoprecipitates (with anti-myc and anti-invertase antibodies, respectively), followed by a second immunoprecipitation with an anti-α1, 6 mannose residue antibody. Unlike invertase, Ero1p-myc could not be reimmunoprecipitated with the anti-α1, 6 mannose residue antibody (compare lanes 2 and 4), indicating that most of the Ero1p protein resided in the endoplasmic reticulum.

Figures 5A and 5B are an autogradiograph and a graph, respectively, showing the regulation of Ero1. **Fig. 5A** shows an autogradiograph of pulse-labeled Ero1p-myc immunoprecipitates treated with 5mM DTT or 10 μg/ml tunicamycin prior to labeling. **Fig. 5B** shows the induction of the unfolded protein response (UPR) (as measured by β-galactosidase activity) by *ero1-1* cultures at 37°C, or by tunicamycin-treated wild-type cultures at 37°C.

Figure 6A is a photograph of plates of cells, as indicated, cultured in the presence of a central filter disk containing 30 μmoles DTT for 3 days at 30°C.

Figure 6B are two graphs showing the growth rate (as measured by OD_{600}) of indicated cells in the presence of 5 mM DTT (upper graph), or 2.5 mM DTT (lower graph).

Figure 6C is a photograph of plates (upper panel) of indicated cells treated for three days with 6 μmoles of diamide at 36°C, and an autoradiograph (lower panel) of Ero1p immunoprecipitates from diamide-treated indicated cells.

Figure 6D is a diagram summarizing the response of cells with either increased or decreased levels of Ero1 function to either DTT or diamide.

Figure 7A is a photograph of plates of ERO1-Δ (CKY563) and PDI1-Δ (CKY564) mutant cells cultured on 5-fluoro-orotic acid (5-FOA) plates in the presence of a central filter disk containing 6 μmoles diamide for 7 days at 25°C.

Figure 7B is a photograph of *ero1-1* mutants (strain CKY559) transformed with the indicated vectors cultured on a YPD plate at 38°C.

Figure 7C is a photograph of PDI1-Δ (CKY564) mutants transformed with the indicated vectors cultured on 5-fluoro-orotic acid (5-FOA) plates for 4 days at 30°C.

Figure 8A is a schematic diagram of the Ero1 protein. The signal sequence peptide is shown in black at the N-terminus, and the four regions of high sequence conservation across species are shown in black. The eight predicted N-linked glycosylation acceptor sites (i.e., Asn X Ser/Thr sites) are as indicated.

Figure 8B is a sequence alignment showing the comparison of the four regions of high sequence conservation in Ero1 with related Ero1 sequences. The species of origin and accession numbers for the sequences are: Sc, *Saccharomyces cerevisiae* (GenBank Accession No.: Z50178); Sp, *Schizosaccharomyces pombe* (GenBank Accession No.: X61926); Tb, *Trypanosoma brucei* (GenBank Accession No.: X60951); Bm, *Brugia malayi* (GenBank Accession No.: AA509062); Dm, *Drosophila melanogaster* (GenBank Accession No.: AA202720); At, *Arabidopsis thaliana* (GenBank Accession No.: T45661); and Hs, *Homo sapiens* (GenBank Accession Nos.: R07093, AA186803, R50884, and AA033538).

Figure 9 is a table listing the yeast strains used herein.

Figure 10 is the consensus nucleic acid (SEQ ID NO: 28) and amino acid (SEQ ID NO: 29) sequence for a mammalian ERO1 cDNA molecule.

Detailed Description

Described below is a novel protein initially isolated from yeast, and termed "Ero1," which is involved in catalyzing the proper formation of disulfide bonds and which may work together for this purpose with another enzyme, PDI, in eukaryotic cells. As described in more detail below, the Ero1 gene was discovered in a screen for new mutations that affected protein secretion in *S. cerevisiae,* and its protein product was subsequently found to be a luminal ER protein essential for disulfide bond formation in the ER. Ero1 appears to be present in many, if not all, eukaryotic cells, since genes homologous to Ero1 exist in other microorganisms (for example, *S. pombe* and *T. brucei*), in plants (*A. thaliana*), and in humans. Ero1 is unique in its ability to vary the oxidizing potential of the ER. In particular, as shown herein, increasing the level of Ero1 increases the oxidizing potential of the ER, and decreasing the level of Ero1 decreases the oxidizing potential of the ER.

Because Ero1 proteins are essential for proper disulfide bond formation, these proteins are useful for catalyzing disulfide bond formation and may be used, if desired, in conjunction with PDI. Ero1 catalysis of disulfide bond formation may be carried out either in vivo or in vitro.

The following examples are included for the purpose of illustrating, and not limiting, the invention.

Identification of Yeast Ero1

To isolate new secretion genes, a collection of 1200 temperature-sensitive *S. cerevisiae* mutants (Hartwell et al., Genetics 74:267, 1973) was screened for defects in protein transport from the ER. To conduct the screen, each mutant was analyzed by Western blotting analysis for the presence of the ER-retained form of carboxypeptidase Y (CPY). The results of this screen

yielded several mutants that displayed defects in ER to Golgi transport that complemented all readily available secretion mutants. One of these new mutations, Ero1, failed to grow above 35°C and exhibited a complete block in the maturation of the ER form of CPY at these elevated temperatures. In tetrad analysis of backcrosses to wild-type strains, the CPY transport defect and temperature sensitivity cosegregated in 2:2 fashion, indicating that both traits were the result of a single nuclear mutation, which we initially designated "sec81-1," and which we now designate "Ero1."

A library of *S. cerevisiae* genomic DNA in the centromere vector YCp50 (Rose et al., Cell 57: 1211-1221, 1989) was screened for plasmids that could rescue the temperature sensitivity of *ero1-1*. In a screen of 20,000 clones, one was identified that complemented both the temperature sensitivity and secretion defects of Ero1. DNA sequencing showed that this clone was derived from the left end of chromosome XIII. Tests of subclones of this region identified the open reading frame YML130c as the complementing gene. An integrating vector with URA3 as a selectable marker was integrated at the YML130c locus by homologous recombination, and, in a cross to an *ero1-1* mutant, the URA3 marker was found to be completely linked to temperature sensitivity identifying YML130c as the Ero1 gene.

A chromosomal deletion of Ero1 was constructed in a diploid strain by one-step gene replacement with a DNA segment with the entire coding sequence of Ero1 replaced with the LEU2 gene. Sporulation of this diploid at 25°C gave only tetrads with two viable spores, neither of which carried the LEU2 marker. Thus, as expected given the existence of the temperature sensitive *ero1-1* mutation, the Ero1 gene was essential for yeast viability. The nucleotide sequence of the coding strand of the Ero1 DNA (SEQ ID NO: 1) is shown in Fig. 1A.

The Ero1 gene encodes a protein with a predicted molecular weight of 56 kD. Overall, the amino acid sequence (SEQ ID NO: 2), which is shown in Fig. 1B, is quite hydrophilic. However, the amino terminus appears to be sufficiently hydrophobic to encode a signal sequence. Searches of GenBank identified proteins with similar sequences to Ero1 in other eukaryotic organisms. These organisms included microorganisms (e.g., *S. pombe* and *T. brucei*), plants (e.g., *A. thaliana*), and mammals (e.g., humans). Shown in Fig. 8A is a schematic diagram of Ero1. The four regions of high sequence conservation to Ero1 proteins found in other species are shown in gray, while the N-terminal signal sequence peptide fragment is shown in black. Also in Fig. 8A are indicated the eight predicted N-linked glycosylation addition sites (i.e., the sites with the sequence Asn X Ser/Thr).

Shown in Fig. 8B is a comparison of the regions 1-4 depicted in Fig. 8A aligned with translations of all of the sequences related to Ero1 found in other eurkaryotes. Of particular note are the conserved regions corresponding to amino acids 164-209 (Region 2 in Fig. 8A) and 325-375 (Region 4 in Fig. 8A) of Ero1, where more than 70% of the residues are identical between species.

Ero1 Is Required for Disulfide Bond Formation

Initially, the *ero1-1* mutation appeared to have a phenotype similar to that of SEC gene mutations that are blocked in ER to Golgi transport. However, tests of different secretory proteins revealed that *ero1-1* blocked the transport of some secretory proteins but not others. In a pulse-chase experiment designed to follow the maturation of CPY, an *ero1-1* mutant at the restrictive temperature of 37°C exhibited a complete block throughout the duration of a 60 minute chase in the conversion of CPY from the ER form (p1)

to the Golgi form and finally, to the vacuolar form (m) of the protein. In a wild-type strain under the same conditions, conversion of the CPY p1 form to the m form occurred in less than 10 minutes (Fig. 2A). In contrast, transport of the secretory protein invertase from the core-glycosolated ER form to the mature secreted form occurred rapidly for both wild type and *ero1-1* strains at 37°C (Fig. 2C).

This finding demonstrated that the *ero1-1* mutation did not interfere with the function of COPII transport vesicles, which are responsible for the transport of both invertase and CPY from the ER. Rather, the ERO1 mutations appeared to selectively impede the ability of CPY to exit the ER. In view of this result, it appeared likely that the *ero1-1* mutation might interfere with disulfide bond formation in the ER. In particular, CPY requires disulfide bond formation in order to exit the ER, whereas invertase does not. When yeast cells are exposed to the reducing agent, DTT, disulfide bonds do not properly form in CPY, and consequently the incorrectly folded protein fails to be transported from the ER. Invertase, in contrast, can fold properly in a reducing environment, as shown by the formation of an enzymatically active cytosolic form of the enzyme and by the ability of invertase to be secreted rapidly even in the presence of DTT. We reproduced these observations, showing that, in wild-type strains exposed to 5 mM DTT in the growth medium, ER transport of CPY, but not invertase, was blocked (Figs. 2A and 2C).

In addition, we tested the effect of *ero1-1* on the transport of the GPI-linked cell surface protein Gas1p. The conversion of Gas1p from its ER-form (120 kD) to its cell-surface form (125 kD) can be detected as an increase in molecular weight. In our experiments, we found that, in wild-type cells, Gas1p maturation from the ER form was blocked by addition of DTT to the growth medium (Fig. 2B). This finding suggested that proper folding and

exit of Gas1p from the ER depended upon disulfide bond formation between cysteine residues in the luminal domain of Gas1p. In an *ero1-1* mutant at 38°C, Gas1p remained in the ER form, co-migrating with a form of the protein produced in an ER to Golgi transport mutant *sec12-4* (Fig. 2B). Thus, the Ero1 mutant exhibited a similar effect on secretory protein exit from the ER as did treatment of wild-type cells with DTT, suggesting that Ero1 played a role in oxidative protein folding in the ER.

To examine disulfide bond formation in the ER, the redox state of CPY was determined in Ero1 mutants. On nonreducing SDS gels, reduced CPY can be resolved from properly folded, oxidized CPY by a difference in mobility. CPY isolated from an *ero1-1* mutant on a nonreducing gel comigrated with reduced CPY produced by treatment of wild-type with DTT (Fig. 3A). Both forms of CPY migrated more slowly than CPY produced in a *sec12* mutant where CPY should be properly oxidized and folded but withheld in the ER where it remains in the p1 form (Fig. 3A, lane 2).

As an independent assay for disulfide bond formation in CPY, we evaluated reactivity of p1 CPY with the thiol-modifying reagent AMS. The maleimide moiety of AMS reacts with cystine thiols on proteins, increasing the molecular weight of the modified protein by approximately 0.5 kD for each AMS residue added. When Ero1 mutant cells were lysed in the presence of AMS, the apparent molecular weight of CPY increased by 5 kD, consistent with the addition of 10 AMS residues (Fig. 3B). In control experiments, p1 CPY prepared from a *sec12* mutant was not detectably modified by AMS, whereas p1 CPY prepared from the same cells that had been treated with 5 mM DTT appeared to be fully modified by AMS. Together, these results indicated that disulfide bonds did not form in CPY expressed in the Ero1 mutant at its restrictive temperature. The secretion block in Ero1 mutants could thus be

explained as a consequence of this defect in oxidative protein folding.

Ero1p Is a Stress-Induced Luminal ER Protein

To detect the Ero1 gene product, we placed a myc epitope at the carboxy terminus of the Ero1 protein coding sequence. ERO1-myc complemented the *ero1*-Δ strain, showing that the modified protein was functional. Antibody to the myc epitope recognized a protein of 96 kD from cells expressing ERO1-myc from a centromere plasmid (Fig. 4A, lane 2). The abundance of Ero1p-myc was greater in cells expressing ERO1-myc from a high copy 2μ plasmid (Fig. 4A, lane 3). Treatment of extracts with endoglycosidase H (Endo H) reduced the apparent molecular weight of Ero1p-myc to 81 kD (Fig. 4A, lane 4); the shift in molecular weight after removal of N-linked carbohydrate chains was consistent with the modification of all eight predicted Asn X Ser/Thr acceptor sites in Ero1p. These experiments indicated that Ero1p was an N-linked glycoprotein, and that the hydrophobic amino terminal sequence was likely a signal sequence.

In addition, Ero1p-myc could not be re-immunoprecipitated with antibody against α1, 6 mannose residues, a modification specific for the cis-Golgi, showing that most of the protein resided in the ER (Fig. 4B). The ERO1 sequence did not contain obvious transmembrane sequences or retention motifs (for example, KKXX or HDEL) raising the question of how the protein was retained in the ER. We found that all of the Ero1p was included in the membrane fractions of cell extracts and could be solubilized by 1% Triton X-100. However, Ero1p-myc was not extracted by 0.5 M NaCl, 2.5 M urea, or 0.1 M carbonate (pH 11.5), conditions that release the luminal protein Kar2p from the membrane. These observations indicated that Ero1p-myc was tightly bound to the inner face of the ER membrane.

In yeast, ER chaperones are regulated by the unfolded protein response (UPR). For example, KAR2 and PDI1 are transcriptionally induced in response to agents that disrupt the maturation of proteins in the ER, such as tunicamycin and DTT. Likewise, Ero1p-myc expression was induced 20-fold by treatment of cells with 5 mM DTT and 10-fold by treatment with 10 μg/ml tunicamycin (Fig. 5A). In cells with ERO1-myc on a multi-copy plasmid, Ero1p-myc expression was increased further upon treatment with DTT or tunicamycin (Fig. 5A, lanes 2μ lanes). Induction of the UPR requires the ER transmembrane kinase encoded by the IRE1 gene. In our experiments, cells with a chromosomal deletion of IRE1 failed to induce expression of ERO1 in response to either DTT or tunicamycin (Fig. 5A, ire1Δ lanes). Thus, ERO1 appeared to be regulated by the established UPR pathway.

We next determined if loss of ERO1 function could induce the UPR. An Ero1 mutant (i.e., *ero1-1*) incubated at either 30°C (permissive) or 37°C (restrictive) induced expression of a UPRE-LacZ reporter about 5-fold as compared to an isogenic wild-type strain under the same conditions (Fig. 5B). A similar induction of the UPRE-LacZ reporter was observed for wild type cells treated with 2.5 μg/ml tunicamycin at 37°C. The compensatory induction of the UPR in *ero1-1* mutants was apparently necessary for cell survival, since in genetic crosses *ero1-1*, *ire1*Δ double mutant segregants were inviable. Both the induction of ERO1 with the UPR and the induction of the UPR in Ero1 mutants supported the role of ERO1 in oxidative protein folding in the ER.

Interaction Between ERO1 and Exogenous Reductants and Oxidants

A mutant with reduced capacity to oxidize protein thiols would be expected to exhibit heightened sensitivity to membrane permeant reducing reagents. We tested the sensitivity of yeast strains to a gradient of DTT

concentrations by placing a filter disk containing 30 μmoles of DTT on top of a lawn of yeast cells. At the permissive temperature of 30°C, an *ero1-1* strain was particularly sensitive to DTT, giving a halo of inviable cells of approximately 4 cm around the disk, whereas an isogenic wild-type strain had a halo of approximately 2 cm in diameter (Fig. 6A). The sensitivity of *ero1-1* mutants to DTT was corroborated by growth assays in liquid culture. Addition of 2.5 mM DTT to YPD slowed the growth of wild-type, but completely prevented the growth of the ero1 mutant (Fig. 6B). The increased sensitivity of the *ero1-1* mutant to DTT at 30°C indicated that, even at permissive growth temperature, this mutant had a reduced capacity to generate an oxidizing environment in the ER, and this result was consistent with the finding that an ER stress response was induced in *ero1-1* at 30°C.

We also tested the possibility that increased dosage of ERO1 could render cells more resistant to DTT. As indicated by the halo assay, a strain overexpressing ERO1 from a high copy plasmid (2μ ERO1) exhibited increased resistance to DTT, producing a halo that was 1.5 cm in diameter (as compared to a halo of 2 cm for wild-type) (Fig. 6A). In corresponding assays in liquid culture, wild type cells were sensitive to 5 mM DTT in YPD, whereas the strain overexpressing ERO1 was capable of growth in this medium (Fig. 6B).

We also carried out tests for sensitivity to DTT in strains carrying a chromosomal deletion of the IRE1 gene. We knew that exposure of yeast cells to DTT induced the UPR, which in turn increased expression of a number of ER proteins, any of which could contribute to the cell's resistance to DTT. The advantage of using an *ire1*-Δ strain background was the elimination of UPR induction, allowing the singular contribution of ERO1 to DTT resistance to be assessed. As was shown previously (Cox et al., Cell 87:391-404, 1996),

deletion of IRE1 increased sensitivity to DTT. Consistent with this result, the halo for an *ire1*-Δ mutant (ire-Δ) was approximately 3 cm, compared to an approximately 2 cm halo for the corresponding wild-type strain (Fig. 6A). Addition of ERO1 on a high copy plasmid to the *ire1*-Δ strain (ire-Δ 2μ ERO1) increased resistance to DTT to about the level of wild type cells, as indicated by both the halo assay and growth in liquid medium supplemented with DTT (Figs. 6A and 6B). From these experiments, it appeared that Ero1p was the limiting component that allowed cells to cope with the stress of lethal doses of DTT, and that the natural resistance of cells to DTT that is afforded by induction of the UPR could be accounted for by an increase in ERO1 expression.

Given that the *ero1-1* mutant increased the sensitivity of cells to exogenous reductant, we explored the possibility that the lethality of Ero1 mutations could be compensated for by an exogenous oxidant. The diazine compound diamide drives formation of disulfide bonds and, when added to the growth medium, enters living cells. We tested the ability of diamide to rescue an *ero1-1* mutation by placing 6 μmoles of diamide on a filter disk onto a lawn of *ero1-1* cells plated at the restrictive temperature of 37°C. The presence of diamide supported a ring of growth of the *ero1-1* mutant, showing that the lethal effect of the mutant was reversed by an appropriate concentration of the oxidant (Fig. 6C). The inner diameter of the ring of growth indicated sensitivity of cells to high concentrations of diamide, which was approximately the same for *ero1-1* and wild-type cells (Fig. 6C, upper panel). To show that diamide could restore the capacity for oxidative folding to Ero1 mutants, we demonstrated that CPY was transported to the vacuole normally when the *ero1-1* mutant cultured at restrictive temperature was exposed to 5 mM diamide in the growth medium (Fig. 6C, lower panel). Finally we tested the ability of

diamide to restore growth to an Ero1 null mutant. A strain containing a chromosomal *ero1*-Δ covered by a functional copy of ERO1 on a URA3 bearing plasmid, was grown without selection in medium containing 5 mM diamide to allow loss of the plasmid. To select for cells that had lost the plasmid but could grow because of an appropriate concentration of exogenous diamide, the culture was plated on medium containing 5-FOA, to select against $Ura3^+$, with 6 μmoles of diamide in a filter disk placed on the lawn to provide a gradient. A ring of clones around the filter disk indicated that the *ero1*-Δ strain could be suppressed by an appropriate concentration of diamide (Fig. 7A). We verified that the colonies growing on this plate carried only the *ero1*-Δ allele by showing that they were *ura3*$^-$ and were dependant on diamide for growth.

The diagram in Figure 6D summarizes the response of cells with either increased or decreased levels of ERO1 function to either exogenous reductant or oxidant. All of the results supported the view that the level of ERO1 function set the redox potential of the ER: increased ERO1 function appeared to make the ER more oxidizing, leading to increased resistance to DTT, whereas reduced ERO1 function appeared to render the ER insufficiently oxidizing, a condition that could be corrected by exogenous diamide.

ERO1 and PDI1 Perform Distinct Functions

Yeast contains a family of genes, related to PDI, that are known to be involved in the proper formation of disulfide bonds. These include: PDI1, EUG1 (Tachibana and Stevens, Mol. Cell Biol. 12:4601, 1992), and MPD1 (an uncharacterized open reading frame which encodes a membrane protein with a PDI-like sequence in what is predicted to be a luminal domain). Although the sequence of ERO1 did not have a thioredoxin motif, common to all PDI-like proteins, we were interested in exploring the functional relationship of Ero1 to

members of the PDI family.

We first tested to see if a reduction in PDI1 function would exacerbate the growth defect of an *ero1-1* mutation. A useful form of PDI1 for such tests is an allele with a deletion of the carboxy terminal HDEL sequence. Cells carrying this PDI-ΔHDEL are viable but have low intracellular levels of Pdi1p because Pdi1p that has escaped the ER cannot be retrieved. In crosses between *ero1-1* and *pdi-ΔHDEL* mutants, spore inviability at 24°C segregated as a two-gene trait (that is, dead:viable spore clones showed 2:2, 1:3, and 0:4 segregation patterns). Genotypic analysis of the surviving sister spore clones revealed that the inviable spores were always *ero1-1 pdi-ΔHDEL* double mutants. This synthetic lethal interaction between the ERO1 and PDI1 genes provided evidence that both genes were involved in the same process. This synthetic lethal interaction was not general for all ER chaperones since double mutants of ero1 and kar2-ΔHDEL were viable and had a threshold restrictive temperature that was the same as that for the *ero1-1* single mutant.

Functional redundancy between members of the yeast PDI family has been demonstrated by the ability of overexpression of either EUG1 or MPD1 to suppress the lethality of a chromosomal deletion of PDI1 (Tachibana and Stevens, *supra*). In our experiments, we introduced a plasmid carrying EUG1 expressed from the GAL1 promoter (pCT44) into an *ero1-1* mutant. Expression of EUG1 on galactose medium could not suppress the temperature sensitivity of *ero1-1* (Fig. 7B), but in a control experiment, pCT44 could suppress the lethality of *pdi1-Δ* (Fig. 7C). Similarly, we found that PDI1 expressed from the GAL1 promoter (pCT37) did not alter the temperature sensitivity of an *ero1-1* mutant.

Methods

The methods used for carrying out the isolation and characterization of Ero1 were as follows.

Plasmid construction

pAF9, isolated from the YCp50 library, carries the ERO1 gene on a 6.5 kb genomic insert. For integrative mapping of the cloned gene, plasmid pAF23, which carries a 2.6 kb *Sal*I-*Xba*I fragment from pAF9 inserted into the integrating vector pRS306, was used. An epitope tagged version of Ero1 was constructed by first introducing a *Not*I site after the last codon of Ero1 by site directed mutagenesis and then inserting a 128 bp sequence that encodes three tandem copies of the c-myc epitope: EQKLISEEDLN (SEQ ID NO: 11). This segment was reconstructed with the full-length gene (including 1156 bp 5' of the ATG and 394 bp 3' of the stop codon) in vector pRS316 to generate pAF82. Ero1-myc was shown to complement both *ero1-1* and *ero1-Δ::LEU2*. The insert with ERO1-myc from pAF82 was inserted into the following vectors: pRS315 (LEU2) to generate pAF85; pRS306-2μ (2μ URA3) to generate pAF84; and pRS305-2μ (2μ LEU2) to generate pAF89. The UPRE reporter pCF118 carries the 5' region of KAR2 fused to lacZ in a CEN LEU2 vector. pNV31 carries the TPI1 promoter fused to the SUC2 gene in a CEN URA3 vector. pAF92 carries the PDI1 gene isolated by PCR amplification of genomic sequences fused to the pGAL1 promoter in vector pCD43 (CEN URA3). This construct was shown to overproduce Pdi1p by Western blotting with anti-Pdi1p antibody. pCT37 carried a fusion of pGAL1 to PDI1 in a CEN URA3 vector and PCT44 carries a fusion of pGAL1 to EUG1 in YEp351 (2μ LEU2).

Media and Strains

A table listing different *S. cerevisiae* strains used in this study is provided in Fig. 9. *S. cerevisiae* cultures were grown and genetically manipulated using techniques as previously described (see, e.g., Kaiser et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1994). The following medias were used to propagate the yeast cultures: YPD, a rich medium with 2% glucose; YEP, a rich medium to which a specified carbon source was added; SD, a minimal medium (Difco Laboratories Inc., Detroit, MI); SC, a minimal medium supplemented with all of the amino acids; and SM, a minimal medium supplemented with adenine plus tryptophan, histidine, arginine, methionine, tyrosine, leucine, isoleucine, lysine, phenylalanine, glutamic acid, aspartic acid, valine, threonine, and serine.

The *ero1-1* mutant was identified in a collection of temperature sensitive mutants in the *S. cerevisiae* strain A364A. The original mutant which was both temperature sensitive and defective in the maturation of CPY was backcrossed 10 times to a wild-type genetic background S288C. In tetrad analysis of the final backcrosses, the CPY transport defect segregated 2:2 and cosegregated with temperature sensitivity. The *ero1-1* mutants were viable at 25°C, grow poorly at 33°C, and were dead at 36°C. CKY558 and CKY559 were two of those backcrossed strains. To disrupt the ERO1 gene, a 1.1 kb *Bgl*II-*Hind*III fragment (encoding amino acids 124-500 of Ero1p) was removed from pAF23 and was replaced by the LEU2 gene from pJJ252 (Jones and Prakash, Yeast 6: 363-366, 1990). A 2.8 kb fragment carrying ero1-Δ::LEU2 was liberated from the resulting plasmid pAF25, by digestion with *Xho*I and *Not*I. CKY562 was constructed by introduction of this ero1-Δ::LEU2 fragment into the chromosome of a diploid formed by mating CKY8 with CKY10 by transformation and homologous recombination. Sporulation of and tetrad

analysis of CKY562 gave 2:2 segregation of lethality where all viable spore clones were Leu-. CKY562 was transformed with pAF82 (ERO1-myc CEN URA3) and on sporulation Leu+ segregants could be isolated, but these depended on pAF82 for viability (no Ura- segregants could be isolated on 5-fluoro-orotic acid plates). A chromosomal deletion of IRE1 was constructed by transformation of CKY10 with the ire1-Δ::URA3 fragment obtained from pCS109A (Shamu and Walter, EMBO J. 15: 3028-3039, 1996). The phenotype of ire1-Δ was verified by the inability of URA+ disruptants to induce LacZ expression from pCF118.

Radiolabeling and Immunoprecipitations

Strains were grown in SD medium containing 2% glucose and auxotrophic supplements to about 1 x 10^7 cells/ml and then were collected by centrifugation and suspended in SD at 1.5 x 10^7 cells/ml. Cell proteins were labeled by addition of 40 μCi of [^{35}S] methionine and cysteine (NEN-Dupont) per 2 x 10^7 cells for 7 minutes. The chase was initiated by addition of 0.3 mM methionine, 0.3 mM cysteine, and 1 mM ammonium sulfate, and samples of 2 x 10^7 cells were collected at times after the initiation of chase in 10 mM NaN_3. Protein extracts were prepared from cell pellets in 30 ml of 80 mM Tris-HCl pH 6.8, 2% β-mercaptoethanol, 2% SDS, and 1 mM PMSF by heating to 95°C for 2 minutes followed by vigorous agitation with acid-washed glass beads. Solubilized samples were suspended in 1ml of IP buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100) preadsorbed with fixed *Staphlococcus A* cells, cleared by centrifugation and then incubated with primary antibody for 2 hours at 25°C. Immune complexes were collected by incubation with Protein A Sepharose (Pharmacia), washed in IP buffer and then solubilized in 20 μl ESB (80 mM Tris-HCl pH 6.8, 100 mM DTT, 1% SDS, 1 mM PMSF, 10%

glycerol, 0.1% bromphenol blue). Samples were resolved by SDS-PAGE and were either exposed to X-ray film (Kodak) or were analyzed with a 445si Phosphorimager and ImageQuant Software (Molecular Dynamics, Sunnyvale, CA).

In particular cases the protocol was modified. To examine the effect of DTT on protein transport, 5 mM DTT was added to cell cultures 10 minutes before labeling. Temperature sensitive mutants were grown at 25°C and then were shifted to 37°C 5 minutes before labeling. We found that Gas1p that had reached the cell surface was sensitive to proteolytic degradation that was not inhibited by our standard cocktail of protease inhibitors. Therefore, for the kinetic analysis of Gas1p maturation, we used a sec6-1 mutant which blocked fusion of post-Golgi secretory vesicles with the plasma membrane, thereby stabilizing the mature form of Gas1p. For detection of intracellular and extracellular invertase (see, e.g., Fy2c), cells were collected in 10 mM NaN_3 and then were converted to spheroplasts by incubation in 0.1 M Tris SO_4 (pH 9.4) and 50 mM β-mercaptoethanol for 10 minutes followed by incubation in 80 µl of 10 mM Tris HCl pH 7.5, 1.2 M sorbitol, and 50 U of recombinant lyticase for 60 minutes at 30°C. Spheroplast pellet and supernatant fractions were separated by centrifugation at 2,500 rpm in a clinical centrifuge. Each fraction was then suspended in IP buffer and incubated with anti-invertase antibody.

Assay of Disulfide Bond Formation in CPY

Disulfide bond formation in CPY was assayed in two different methods. The first method relied on the fact that properly folded, oxidized CPY migrates more rapidly than reduced CPY on nonreducing SDS PAGE.

Wild type (CKY10), *ero1-1* (CKY559), and *sec12* (CKY39) strains were grown in SD medium at 25°C then shifted to 38°C for 20 minutes. To half of the samples 5 mM DTT was added, and incubation was continued for 10 minutes. The cells were then labeled with [^{35}S] methionine and cysteine for 30 minutes at 38°C. Labeling was terminated, and free thiols were blocked by placing cells in 10 mM NaN_3 and 20 mM N-ethyl maliemide (NEM). Cell pellets were lysed in either 30 µl of 80 mM Tris-HCl pH 6.8, 2% β-mercaptoethanol, 2% SDS, and 1 mM PMSF (reducing) or 30 µl of the same buffer without β-mercaptoethanol (nonreducing). CPY was immunoprecipitated, and samples were suspended in either ESB, or ESB without DTT, and resolved by SDS-PAGE.

In the second method, free protein thiols were modified with AMS, and the extent of modification was detected as a decrease in mobility on SDS-PAGE. Wild type (CKY10), *ero1-1* (CKY559), and *sec12* (CKY39) strains were grown in SD medium at 25°C, shifted to 38°C for 10 minutes, and then labeled with [^{35}S] methionine and cysteine for 30 minutes at 38°C. To half of the samples 5 mM DTT was added 10 minutes before labeling. Cell pellets were lysed in 30 µl of 80 mM Tris-HCl pH 6.8, 1% SDS, 1 mM PMSF, and 20 mM AMS, and were incubated in this buffer for 30 minutes at 25°C. CPY was immunoprecipitated and resolved on nonreducing gels as described above.

Detection and Quantitation of Ero1p

To detect Ero1p, strains expressing a myc-tagged version of the gene (ERO1-myc) were grown in SD medium at 30°C, and were then pulse labeled with [^{35}S] methionine and cysteine for 30 minutes. Cell pellets were lysed in

30 µl of 80 mM Tris-HCl pH 6.8, 20 mM β-mercaptocthanol, 1% SDS, 1 mM PMSF, and diluted into 1 ml of IP buffer. Ero1p-myc was immunoprecipitated with 9E10 monoclonal anti-myc antibody, suspended in ESB, and resolved by SDS-PAGE. For endoH digestion samples were diluted four-fold in 50 mM sodium citrate pH 5.5 with 100 units of endoH (New England Biolabs). To test induction of Ero1 expression by the UPR, either 5 mM DTT or 10 µg/ml of tunicamycin (Sigma) was added to the cultures 10 minutes before labeling. Quantitation of Ero1 induction was analyzed with a phosphorimager.

To test for the Golgi-specific α 1,6 mannose modification on Ero1p, immunoprecipitates of Ero1p-myc were reprecipitated with anti-α 1,6 mannose antibody. As a control to demonstrate the efficacy of the antibody, mature invertase (isolated from a sec6 mutant) was precipitated with anti-invertase antibody and then reprecipitated with anti-α 1,6 mannose antibody.

Induction of the Unfolded Protein Response

CKY10 (ura3-52) and CKY559 (ero1-1 ura3-52) were transformed with the plasmid pCF118, which carries a kar2-lacZ fusion. Transformants were grown in SC medium without leucine at 25°C to a density of 1 x 10^7 cells/ml. The temperature was then shifted to either 30°C or 37°C, or 2.5 µg/ml tunicamycin was added, and incubation was continued for 2.5 hours. Cells were then permeabilized by treatment with chloroform and SDS, and β-galactosidase activity was assayed by standard techniques. Enzymatic activities were normalized to OD_{600}. Two transformants were assayed, and the experiment was repeated twice.

To test for synthetic-lethality between *ero1-1* and *pdi1-ΔHDEL*, strains CKY558 and CKY395 were crossed and the resulting tetrads were dissected. Lethality segregated as a two-gene trait on YPD medium at 25°C

(segregation patterns of live: dead of 4:0, 1:3, and 2:2 were seen). The surviving sister spores were tested for ero1-1 (temperature sensitivity) and pdi1-ΔHDEL (Pdi1p secretion into the medium), and their genotypes showed that the dead spores were always double mutants.

Growth Tests in the Presence of DTT or Diamide

To test for sensitivity to DTT, 2 x 10^6 cells were plated on YPD medium and 30 μ moles of DTT was placed on top of the lawn in a 6 mm sterile filter disk. Plates were photographed after three days at 30°C. Growth was also tested by measuring the change in OD_{600} with time in YPD medium with either 2.5 mM DTT or 5 mM DTT. To test for rescue by diamide, wild-type (CKY10) or *ero1-1* (CKY559) were plated at 3 x 10^6 cells/plate and 6 μ moles of diamide was placed on top of the lawn in a 6 mm sterile filter disk. Plates were photographed after three days at 36°C. As negative controls, temperature-sensitive kar2 mutants (kar2-159 and kar2-203) were plated at 33°C, and no growth was detected in the presence of diamide. To test for suppression of *ero1*-Δ by diamide, the null allele covered by ERO1 genes on URA3 plasmid (CKY563) was grown to saturation in YPD with 0.4 mM diamide to allow loss of the plasmid. Similarly, the plasmid covered *pdi*-Δ strain (CKY564) was grown in YEP medium with 2% galactose and 0.4 mM diamide. Cultures were plated at 3 x 10^6 cells/plate onto SC plates supplemented with 1 mg/ml 5 fluoro-oritic acid and a filter disk with 6 μmoles of diamide. The plates were photographed after seven days at 25°C. The colonies of CKY563 that grew in a ring around the diamide source were shown to be diamide dependent (on replating on a SC plate with 6 μmoles of diamide spotted in the center, they grew only near the source of diamide), and ura3- (they did not grow on SC plates without uracil even in the presence of

diamide).

Suppression Tests

To test the ability of overexpression of PDI1 or EUG1 to suppress the temperature sensitivity of *ero1-1*, CKY559 was transformed with pAF82 (ERO1-myc CEN URA3), pAF92 (pGAL1-PDI1 CEN URA3), pCT44 (pGAL1-EUG1 2μ LEU2), or prs316 (CEN URA3). Purified transformants were grown selectively on SC medium, and then in SC medium with 2% raffinose and 2% galactose to induce pGAL1 expression. Serial dilutions of the cultures were spotted on YEP medium with 2% raffinose and 2% galactose and incubated at 38°C for three days. To confirm that EUG1 overexpression suppressed the lethality of pdi1-Δ, CKY564 (pdi1-Δ [pGAL1-PDI1 URA3]) and CKY564 with pCT44 were plated on SM medium containing 2% raffinose and 2% galactose and 1 mg/ml 5-FOA. These plates were incubated at 30°C for four days.

To test for the ability of overexpression of ERO1 to suppress kar2 mutations, CKY222 (kar2-159) and CKY229 (kar2-203) were transformed with pAF84 (ERO1-myc 2μ URA3), and were plated at restrictive temperatures of 30°-38°C. No improvement of growth of the transformed strains was detected over strains transformed with vector only.

Cloning Mammalian Ero1 Sequences

Based on our isolation of novel yeast ERO1 cDNAs, the isolation of mammalian ERO1 nucleic acid sequences, including human ERO1 sequences, is made possible using the sequences described herein and standard techniques. In particular, using all or a portion of a yeast ERO1 sequence, one may readily design oligonucleotide probes, including degenerate oligonucleotide probes

(i.e., a mixture of all possible coding sequences for a given amino acid sequence). These oligonucleotides may be based upon the sequence of either strand of the DNA.

Exemplary probes or primers for isolating mammalian ERO1 sequences preferably correspond to conserved blocks of amino acids, for example, conserved Ero1 motifs. Exemplary motifs are as follows, in the N to C direction, using the standard one letter code where (X) is any amino acid, (Ac) is any acidic amino acid, (Ba) is any basic amino acid, and (Hb) is any hydrophobic amino acid:

Ero1 Region 1: LLKSDFFKYFRLDLYKQCSFW (SEQ ID NO: 3);

Ero1 Region 2: AVLIDLTANPERFTGYGGKQAGQIWSTIYQDNC (SEQ ID NO: 4);

Ero1 Region 3: AKDAFYRLVSGFHASIGTHLS (SEQ ID NO: 5);

Ero1 Region 4: LKDEFRSRFKNVTKIMDCVQCDRCRLWGKIQTTG YATALKILF (SEQ ID NO: 6);

Ero1 Region 2A: DL(X)(X)NPE(X)(X)TGY (SEQ ID NO: 7);

Ero1 Region 3A: L(Hb)SGLHASI (SEQ ID NO: 8);

Ero1 Region 4A: (Hb)MDCV(X)C(Ac)(Ba)CR(Hb)WGK (SEQ ID NO: 9); and

Ero1 Region 4B: TALK(Hb)(Hb)F (SEQ ID NO: 10).

Using such motifs, partial or complete mammalian ERO1 genes may be isolated from sequence databases (for example, by the use of standard programs such as Pileup). Examination of the yeast ERO1 sequence, for example, has allowed for the elucidation of the mammalian ERO1 sequence shown in Fig. 10 (SEQ ID NOS: 28 and 29). The nucleotide residues of this sequence were derived from the GenBank sequences listed below in Table I.

Table I

Sources for Mammalian Ero1 Consensus Sequence

Nucleotide Sequence	GenBank Accession No.	Source
1-156	AA305384	human
157-242	AI060157 AA920983 AA867609	rat mouse mouse
243-508	R07093	human
509-805	AA179578	human
806-900	R50884 AA186803	human human
900-1102	AA021774 AA596783 AA896877	mouse mouse mouse
1103-1361	AA356773	human
1362-1781	AA573318 AA179345	human human
1782-1792	AA186804	human
1793-1848	c18854	human

In an alternative approach to isolating mammalian ERO1 sequences, the motifs described above may be used to design degenerate oligonucleotide probes to probe large genomic or cDNA libraries directly. General methods for

designing and preparing such probes are provided, for example, in Ausubel et al., Current Protocols in Molecular Biology, 1996, Wiley & Sons, New York, NY; and Guide to Molecular Cloning Techniques, 1987, S. L. Berger and A. R. Kimmel, eds., Academic Press, New York. These oligonucleotides are useful for Ero1 gene isolation, either through their use as probes for hybridizing to Ero1 complementary sequences or as primers for various polymerase chain reaction (PCR) cloning strategies. If a PCR approach is utilized, the primers are optionally designed to allow cloning of the amplified product into a suitable vector. PCR is particularly useful for screening cDNA libraries from rare tissue types.

Hybridization techniques and procedures are well known to those skilled in the art and are described, for example, in Ausubel et al., *supra*, and Guide to Molecular Cloning Techniques, *supra*. If desired, a combination of different oligonucleotide probes may be used for the screening of the recombinant DNA library. The oligonucleotides are, for example, labelled with ^{32}P using methods known in the art, and the detectably-labelled oligonucleotides are used to probe filter replicas from a recombinant DNA library. Recombinant DNA libraries (for example, human cDNA libraries) may be prepared according to methods well known in the art, for example, as described in Ausubel et al., *supra*, or may be obtained from commercial sources. Preferred libraries for isolating mammalian Ero1 homologs include, without limitation, human and murine cDNA libraries from various tissues, and human and murine genomic libraries. Such libraries may be generated using standard techniques, and are also commercially available (from, e.g., Clontech Laboratories, Inc.)

For detection or isolation of closely related ERO1 sequences, high stringency hybridization conditions may be employed; such conditions include

hybridization at about 42°C and about 50% formamide; a first wash at about 65°C, about 2X SSC, and 1% SDS; followed by a second wash at about 65°C and about 0.1% SDS, 1X SSC. Lower stringency conditions for detecting ERO1 genes having less sequence identity to the yeast ERO1 genes described herein include, for example, hybridization at about 42°C in the absence of formamide; a first wash at about 42°C, about 6X SSC, and about 1% SDS; and a second wash at about 50°C, about 6X SSC, and about 1% SDS.

As discussed above, ERO1-specific oligonucleotides may also be used as primers in PCR cloning strategies. Such PCR methods are well known in the art and are described, for example, in PCR Technology, H.A. Erlich, ed., Stockton Press, London, 1989; PCR Protocols: A Guide to Methods and Applications, M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White, eds., Academic Press, Inc., New York, 1990; and Ausubel et al., *supra*. Again, sequences corresponding to conserved regions in an Ero1 sequence (for example, those regions described above) are preferred for use in isolating mammalian Ero1 sequences. Such probes may be used to screen cDNA as well as genomic DNA libraries.

Following isolation of such candidate genes by sequence homology, the genes may be tested for their ability to functionally complement a yeast *Ero1* mutation (e.g., *ero1-1*). This is most readily assayed by transformation of the sequence into an Ero1 conditional mutant strain and testing for viability under restrictive conditions. Exemplary yeast transformation techniques are described, for example, in Kaiser et al., Methods in Yeast Genetics, 1994, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, and assays for Ero1 function are described herein. In evaluating sequences by this approach, a mammalian sequence need not fully complement a yeast Ero1 defect, but must preferably provide a detectable level of functional complementation.

Alternatively, a sequence may be tested for function in any standard protein reduction assay, for example, the insulin reduction assay described in Holmgren et al., J. Biol. Chem. 254: 9627-9632, 1979.

Ero1 Polypeptide Expression

In general, Ero1 polypeptides according to the invention may be produced by transformation of a suitable host cell with all or part of an Ero1-encoding cDNA fragment (e.g., one of the cDNAs described herein or isolated as described above) in a suitable expression vehicle.

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems may be used to provide the recombinant protein. The precise host cell used is not critical to the invention. The Ero1 polypeptide may be produced in a prokaryotic host (e.g., *E. coli*) or in a eukaryotic host (e.g., *Saccharomyces cerevisiae*, insect cells, e.g., Sf9 or Sf21 cells, or mammalian cells, e.g., COS 1, NIH 3T3, or HeLa cells). Such cells are available from a wide range of sources (e.g., the American Type Culture Collection, Rockland, MD; also, see, e.g., Ausubel et al., *supra*). The method of transformation or transfection and the choice of expression vehicle will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel et al. (*supra*); expression vehicles may be chosen from those provided, e.g., in Cloning Vectors: A Laboratory Manual (P.H. Pouwels et al., 1985, Supp. 1987).

Alternatively, an Ero1 polypeptide is produced in a mammalian system, for example, by a stably-transfected mammalian cell line. A number of vectors suitable for stable transfection of mammalian cells are available to the public, e.g., see Pouwels et al. (*supra*); methods for constructing such cell lines are also publicly available, e.g., in Ausubel et al. (*supra*). In one example,

cDNA encoding the Ero1 protein is cloned into an expression vector which includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, the Ero1 protein-encoding gene into the host cell chromosome is selected for by inclusion of 0.01-300 μM methotrexate in the cell culture medium (as described in Ausubel et al., *supra*). This dominant selection may be accomplished in most cell types. Recombinant protein expression may be increased by DHFR-mediated amplification of the transfected gene. Methods for selecting cell lines bearing gene amplifications are described in Ausubel et al. (*supra*); such methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. DHFR-containing expression vectors commonly used for this purpose include pCVSEII-DHFR and pAdD26SV(A) (described in Ausubel et al., *supra*). Any of the host cells described above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO DHFR$^-$ cells, ATCC Accession No. CRL 9096) are among the host cells preferred for DHFR selection of a stably-transfected cell line or DHFR-mediated gene amplification.

In yet other alternative approaches, the Ero1 polypeptide is produced *in vivo* or, preferably, *in vitro* using a T7 system (see, for example, Ausubel et al., *supra*, or other standard techniques).

Once the recombinant Ero1 protein is expressed, it is isolated, e.g., using affinity chromatography. In one example, an anti-Ero1 protein antibody (e.g., produced as described herein) may be attached to a column and used to isolate the Ero1 protein. Lysis and fractionation of Ero1 protein-harboring cells prior to affinity chromatography may be performed by standard methods (see, e.g., Ausubel et al., *supra*).

Once isolated, the recombinant protein can, if desired, be further purified, e.g., by high performance liquid chromatography (see, e.g., Fisher,

Laboratory Techniques In Biochemistry And Molecular Biology, eds., Work and Burdon, Elsevier, 1980).

Polypeptides of the invention, particularly short Ero1 polypeptide fragments, may also be produced by chemical synthesis (e.g., by the methods described in Solid Phase Peptide Synthesis, 2nd ed., 1984, The Pierce Chemical Co., Rockford, IL).

These general techniques of polypeptide expression and purification may also be used to produce and isolate useful Ero1 fragments or analogs (described herein).

Anti-Ero1 Antibodies

Using the Ero1 polypeptide described herein or isolated as described above, anti-Ero1 antibodies may be produced by any standard technique. In one particular example, an Ero1 cDNA or cDNA fragment encoding a conserved Ero1 domain is fused to GST, and the fusion protein produced in *E. coli* by standard techniques. The fusion protein is then purified on a glutathione column, also by standard techniques, and is used to immunize rabbits. The antisera obtained is then itself purified on a GST-Ero1 affinity column and is shown to specifically identify GST-Ero1, for example, by Western blotting.

Polypeptides for antibody production may be produced by recombinant or peptide synthetic techniques (see, e.g., Solid Phase Peptide Synthesis, *supra*; Ausubel et al., *supra*).

For polyclonal antisera, the peptides may, if desired, be coupled to a carrier protein, such as KLH as described in Ausubel et al, *supra*. The KLH-peptide is mixed with Freund's adjuvant and injected into guinea pigs, rats, or preferably rabbits. Antibodies may be purified by any method of peptide

antigen affinity chromatography.

Alternatively, monoclonal antibodies may be prepared using an Ero1 polypeptide (or immunogenic fragment or analog) and standard hybridoma technology (see, e.g., Kohler et al., Nature 256:495, 1975; Kohler et al., Eur. J. Immunol. 6:511, 1976; Kohler et al., Eur. J. Immunol. 6:292, 1976; Hammerling et al., In: Monoclonal Antibodies and T Cell Hybridomas, Elsevier, NY, 1981; Ausubel et al., *supra*).

Once produced, polyclonal or monoclonal antibodies are tested for specific Ero1 recognition by Western blot or immunoprecipitation analysis (by the methods described in Ausubel et al., *supra*). Antibodies which specifically recognize an Ero1 polypeptide described herein are considered to be useful in the invention.

Using such techniques, an antibody specific for the yeast Ero1 polypeptide has been isolated.

Use of Ero1 in Eukaryotic Expression Systems

Because of their ability to catalyze disulfide bond formation, Ero1 proteins may be used to improve the yield of properly folded, disulfide bond-containing proteins of interest, for example, commercially important recombinant proteins. Ero1 techniques may be carried out either in vivo or in vitro, and exemplary Ero1-based methods of protein production are now described.

In Vitro Refolding Reactions

In general, this approach involves the use of purified Ero1 in combination with any in vitro refolding reaction. In one particular example, a recombinant protein of interest is expressed (for example, in an *E. coli* or

mammalian cell culture system) and is treated with a denaturant, such as guanidine hydrochloride. The protein preparation is then allowed to refold by dilution of the denaturant, and proper disulfide bond formation is promoted during this renaturation step by the presence of Ero1 protein in the reaction mixture. If desired, the Ero1 protein may be added in a buffer combined with oxidized and reduced glutathione and/or purified PDI.

In Vivo Expression Systems

Ero1 may also be used to catalyze proper disulfide bond formation in any in vivo protein expression system. By this approach, a full-length Ero1-expressing cDNA is introduced into a host cell which also expresses a secreted protein of interest. Preferably, the cDNA encodes the Ero1 protein which corresponds most closely to the protein of interest (for example, human Ero1 is preferably expressed in a cell culture for production of a human protein of interest), and the Ero1 is preferably produced at high levels in the cultured cells. Although mammalian tissue culture cells are preferred for this purpose, any appropriate eukaryotic cell may be used for protein expression in conjunction with an Ero1 product. This technique may be used for the production of any protein which is naturally secreted by a eukaryotic cell or which may be joined to a heterologous signal sequence that artificially directs secretion of the protein from the host cell.

Screens for Compounds that Alter the Oxidizing Potential of the Endoplasmic Reticulum

The Ero1 reagents provided herein facilitate the development of a variety of screens to identify compounds that can alter the oxidizing potential of the endoplasmic reticulum (ER). Compounds that can either increase or

decrease the oxidizing potential of the ER allow for the fine-tuning of an in vivo or in vitro expression system for a particular protein. For example, by carefully modifying the oxidizing potential of the ER in vivo, a cell may be manipulated to selectively over-express a correctly folded recombinant protein, while having a reduced level of expression of an incorrectly folded endogenous protein or a reduced level of formation of insoluble protein aggregates. Likewise, such an oxidizing potential-modifying compound may be added to in vitro expression systems to maximize the correct folding of a denatured protein substrate of interest.

In one particular example of a preferred screen, a yeast cell bearing a temperature sensitive Ero1 mutation, such as the *ero1-1* mutant described above, may be utilized to identify a compound that reduces the oxidizing potential of the ER. In this example, a wild-type yeast and an *ero1-1* mutant yeast are each exposed to a candidate compound and then grown at a temperature which is permissive to both the wild-type and mutant cells. Compounds that result in the death of *ero1-1* mutant yeast cells, but not in the death of wild-type yeast cells, are selected based on their ability to render the *ero1-1* mutant hypersensitive. Such compounds act as inhibitors of Ero1 activity and may be employed to reduce the oxidizing potential of the ER, for example, in in vivo or in vitro expression systems.

Other Embodiments

In other embodiments, the invention includes any protein which possesses the requisite level of amino acid sequence identity (as defined herein) to the yeast Ero1 sequence; such homologs include other substantially pure naturally-occurring mammalian Ero1 polypeptides (for example, human Ero1

polypeptides) as well as allelic variants; natural mutants; induced mutants; proteins encoded by DNA that hybridizes to the ERO1 DNA sequence or degenerate conserved domains of Ero1 proteins (e.g., those described herein) under high stringency conditions; and proteins specifically bound by antisera directed to an Ero1 polypeptide.

The invention further includes analogs of any naturally-occurring Ero1 polypeptides. Analogs can differ from the naturally-occurring protein by amino acid sequence differences which do not destroy function, by post-translational modifications, or by both. Modifications include *in vivo* and *in vitro* chemical derivatization of polypeptides, e.g., acetylation, carboxylation, phosphorylation, or glycosylation; such modifications may occur during polypeptide synthesis or processing or following treatment with isolated modifying enzymes. Analogs can also differ from the naturally-occurring Ero1 polypeptide by alterations in primary sequence. These include genetic variants, both natural and induced (for example, resulting from random mutagenesis by irradiation or exposure to ethanemethylsulfate or by site-specific mutagenesis as described in Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual (2d ed.), CSH Press, 1989, or Ausubel et al., *supra*). Also included are cyclized peptides, molecules, and analogs which contain residues other than L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g., β or γ amino acids.

In addition to full-length polypeptides, the invention also includes Ero1 polypeptide fragments. As used herein, the term "fragment," means at least 20 contiguous amino acids, preferably at least 30 contiguous amino acids, more preferably at least 50 contiguous amino acids, and most preferably at least 60 to 80 or more contiguous amino acids. Fragments of such Ero1 polypeptides can be generated by methods known to those skilled in the art or

may result from normal protein processing (e.g., removal of amino acids from the nascent polypeptide that are not required for biological activity or removal of amino acids by alternative mRNA splicing or alternative protein processing events). For certain purposes, all or a portion of an Ero1 polypeptide sequence may be fused to another protein (for example, by recombinant means).

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

Other embodiments are within the following claims.

What is claimed is:

Claims

1. A method of increasing disulfide bond formation in a protein, said method comprising:

(a) denaturing said protein; and

(b) allowing renaturation of said protein in the presence of an Ero1 polypeptide.

2. The method of claim 1, wherein said Ero1 polypeptide is combined with a protein disulfide-isomerase.

3. A method of increasing disulfide bond formation in a protein, said method comprising expressing said protein in a host cell that also expresses an isolated nucleic acid that encodes an Ero1 polypeptide.

4. The method of claim 3. wherein said host cell further expresses a nucleic acid encoding a protein disulfide-isomerase.

5. The method of claim 1 or 3, wherein said protein is a secreted protein.

6. The method of claim 1 or 3, wherein said Ero1 polypeptide is derived from a yeast.

7. A substantially pure preparation of an Ero1 polypeptide.

8. The polypeptide of claim 7, wherein said polypeptide comprises an amino acid sequence which is at least 27% identical to the amino acid

sequence of SEQ ID NO: 2.

9. The polypeptide of claim 8, wherein said polypeptide comprises an amino acid sequence which is at least 80% identical to the amino acid sequence of SEQ ID NO: 2.

10. The polypeptide of claim 7, wherein said polypeptide comprises an amino acid sequence which is at least 27% identical to the sequence of SEQ ID NO: 29.

11. The polypeptide of claim 7, wherein said polypeptide comprises an amino acid sequence which is at least 50% identical to at least one of the amino acid sequences of SEQ ID NOS: 3-10.

12. The polypeptide of claim 11, wherein said polypeptide comprises an amino acid sequence which is at least 90% identical to at least one of the amino acid sequences of SEQ ID NOS: 3-10.

13. The polypeptide of claim 7, wherein said polypeptide is derived from a mammal.

14. The polypeptide of claim 7, wherein said polypeptide is derived from a yeast.

15. Isolated nucleic acid encoding an Ero1 polypeptide.

16. The nucleic acid of claim 15, wherein said nucleic acid

comprises a nucleic acid sequence which is at least 27% identical to the nucleic acid sequence of SEQ ID NO: 1.

17. The nucleic acid of claim 16, wherein said nucleic acid comprises a nucleic acid sequence which is at least 80% identical to the nucleic acid sequence of SEQ ID NO: 1.

18. The nucleic acid of claim 15, wherein said nucleic acid comprises a nucleic acid sequence which is at least 27% identical to the nucleic acid sequence of SEQ ID NO: 28.

19. The nucleic acid of claim 15, wherein said nucleic acid encodes an amino acid sequence which is at least 50% identical to at least one of the amino acid sequences of SEQ ID NOS: 3-10.

20. The nucleic acid of claim 17, wherein said nucleic acid encodes an amino acid sequence which is at least 90% identical to at least one of the amino acid sequences of SEQ ID NOS: 3-10.

21. The nucleic acid of claim 15, wherein said nucleic acid comprises the sequence of SEQ ID NO: 1.

22. The nucleic acid of claim 15, wherein said nucleic acid comprises the sequence of SEQ ID NO: 29.

23. The nucleic acid of claim 15, wherein said nucleic acid complements an *Ero1* mutation in *S. cerevisiae*.

24. A vector comprising the isolated nucleic acid of claim 15, said vector being capable of directing the expression of the protein encoded by said nucleic acid in a vector-containing cell.

25. A cell comprising the isolated nucleic acid of claim 15 or the vector of claim 24.

26. The cell of claim 25, wherein said cell is a bacterial cell.

27. The cell of claim 25, wherein said cell is a mammalian cell.

28. The cell of claim 25, wherein said cell is a yeast cell.

29. A method of producing a recombinant Ero1 polypeptide comprising the steps of:

(a) providing a cell transformed with an isolated nucleic acid encoding an Ero1 polypeptide positioned for expression in said cell under conditions for expressing said isolated nucleic acid; and

(b) expressing said recombinant Ero1 polypeptide.

30. An Ero1 polypeptide produced according to the method of claim 29.

31. A substantially pure antibody that specifically recognizes and binds an Ero1 polypeptide.

32. The antibody of claim 31, wherein said Ero1 polypeptide is

derived from a yeast.

33. A method of detecting a gene or a portion thereof found in a mammalian cell having sequence identity to the Ero1 sequence of SEQ ID NO: 1 or SEQ ID NO: 28, said method comprising contacting the nucleic acid of claim 15 or a portion thereof greater than about 15 residues in length, or a degenerate oligonucleotide corresponding to at least one of SEQ ID NOS: 3-10, with a preparation of nucleic acid from said mammalian cell under hybridization conditions providing detection of nucleic acid sequences having about 50% or greater nucleic acid sequence identity to the Ero1 sequence of SEQ ID NO: 1 or SEQ ID NO: 28.

34. A method of isolating a gene or a portion thereof found in a mammalian cell having 50% nucleic acid sequence identity to a sequence encoding at least one of SEQ ID NOS: 3-10, said method comprising the steps of:

(a) amplifying by PCR said mammalian gene or portion thereof using oligonucleotide primers wherein said primers

(i) are each greater than about 12 residues in length; and

(ii) each have regions of complementarity to opposite nucleic acid strands in a region of the nucleotide sequence of SEQ ID NO: 1 or SEQ ID NO: 28; and

(b) isolating said mammalian gene or portion thereof.

35. The method of claim 33 or 34, wherein said mammal is a human.

36. The method of claim 33 or 34, wherein said method further comprises a step of testing said gene or portion thereof for the ability to functionally complement a yeast *Ero1* mutant.

Fig. 1A

atgagattaagaaccgccattgccacactgtgcctcacggcttttacatctgcaacttcaaacaatagctacatcgccac
cgaccaaacacaaaatgcctttaatgacactcacttttgtaaggtcgacaggaatgatcacgttagtcccagttgtaacg
taacattcaatgaatttaaatgccataaatgaaaacattagagatgatctttcggcgttattaaaatctgatttcttcaaa
tacttteggctggatttatacaagcaatgttcattttgggacgccaacgatggtctgtgcttaaaccgcgcttgctctgt
tgatgtcgtagaggactgggatacactgcctgagtactggcagcctgagatcttgggtagtttcaataatgatacaatga
aggaagcggatgatagcgatgacgaatgtaagttcttagatcaactatgtcaaaccagtaaaaaaacctgtagatatcgaa
gacaccatcaactactgtgatgtaaatgactttaacggtaaaaacgccgttctgattgatttaacagcaaatccggaacg
atttacaggttatggtggtaagcaagctggtcaaatttggtctactatctaccaagacaactgttttacaattggcgaaa
ctggtgaatcattggccaaagatgcattttatagacttgtatccggtttccatgcctctatcggtactcacttatcaaag
gaatatttgaacacgaaaactggtaaatgggagcccaatctggatttgtttatggcaagaatcgggaactttcctgatag
agtgacaaacatgtatttcaattatgctgttgtagctaaggctctctggaaaattcaaccatatttaccagaattttcat
tctgtgatcctagtcaataaagaaatcaaaaacaaaatggataacgttatttcccagctggacacaaaaatttttaacgaa
gacttagtttttgccaacgacctaagtttgactttgaaggacgaattcagatctcgcttcaagaatgtcacgaagattat
ggattgtgtgcaatgtgatagatgtagattgtggggcaaaattcaaactaccggttacgcaactgccttgaaaatttttgt
ttgaaatcaacgacgctgatgaattcaccaaacaacatattgttggtaagttaaccaaatatgagttgattgcactatta
cagactttcggtagattatctgaatctattgaatctgttaacatgttcgaaaaaatgtacgggaaaaggttaaacggtlc
tgaaaacaggttaagctcattcttccaaaataacttcttcaacatttgaaggaggcaggcaaatcgattcgttacacca
tagagaacatcaattccactaaagaaggaaagaaaaagactaacaattctcaatcacatgtatttgatgatttaaaaatg
cccaaagcagaaatagttccaaggccctctaacggtacagtaaataaatggaagaaagcttggaatactgaagttaacaa
cgttttagaagcattcagatttatttatagaagctatttggatttacccaggaacatctgggaattatctttgatgaagg
tatacaaatttttggaataaattcatcggtgttgctgattacgttagtgaggagacacgagagcctatttcctataagcta
gatatacaataa

Fig. 1B

MRLRTAIATLCLTAFTSATSNNSYIATDQTQNAFNDTHFCKVDR
NDHVSPSCNVTFNELNAINENIRDDLSALLKSDFFKYFRLDLYKQCSFWDANDGLCLN
RACSVDVVEDWDTLPEYWQPEILGSFNNDTMKEADDSDDECKFLDQLCQTSKKPVDIE
DTINYCDVNDFNGKNAVLIDLTANPERFTGYGGKQAGQIWSTIYQDNCFTIGETGESL
AKDAFYRLVSGFHASIGTHLSKEYLNTKTGKWEPNLDLFMARIGNFPDRVTNMYFNYA
VVAKALWKIQPYLPEFSFCDLVNKEIKNKMDNVISQLDTKIFNEDLVFANDLSLTLKD
EFRSRFKNVTKIMDCVQCDRCRLWGKIQTTGYATALKILFEINDADEFTKQHIVGKLT
KYELIALLQTFGRLSESIESVNMFEKMYGKRLNGSENRLSSFFQNNFFNILKEAGKSI
RYTIENINSTKEGKKTNNSQSHVFDDLKMPKAEIVPRPSNGTVNKGKKAWNTEVNNV
LEAFRFIYRSYLDLPRNIWELSLMKVYKFWNKFIGVADYVSEETREPISYKLDIQ

Fig.2 A

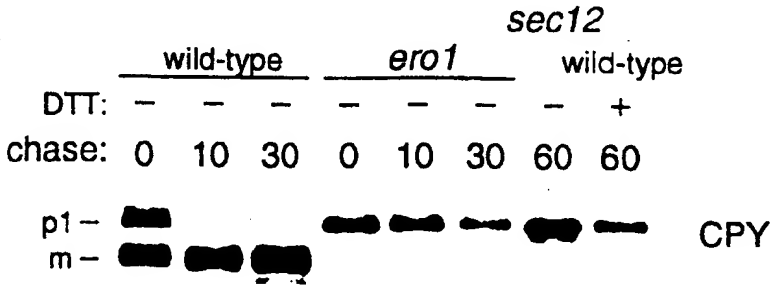


Fig.2 B

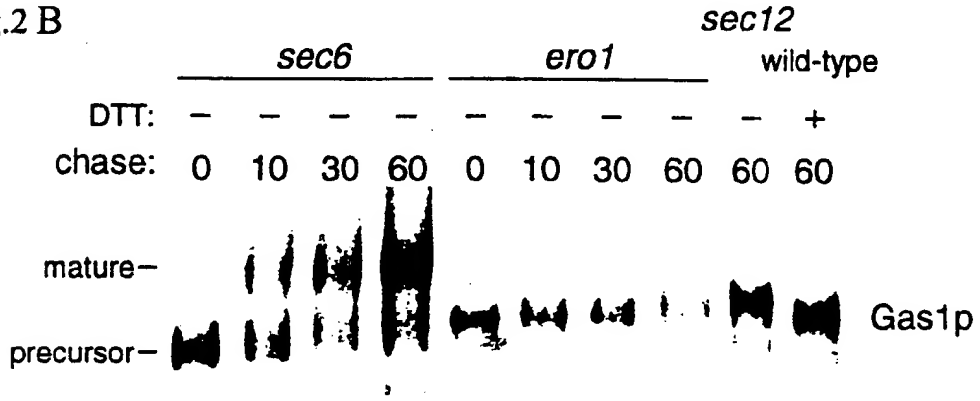


Fig.2 C

Fig.3 A

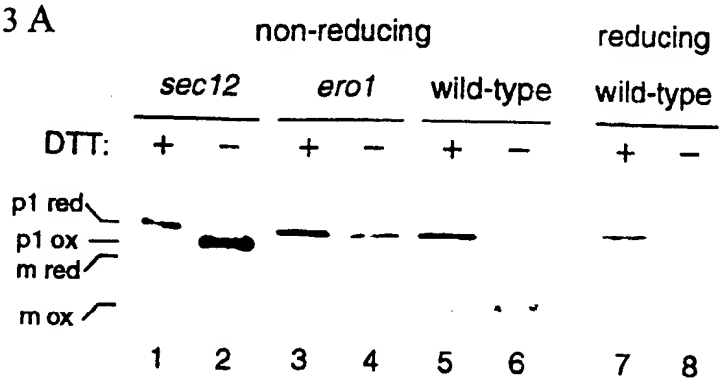

non-reducing
reducing
sec12
ero1
wild-type
wild-type
DTT: + − + − + − + −
p1 red
p1 ox
m red
m ox
1 2 3 4 5 6 7 8

Fig.3 B

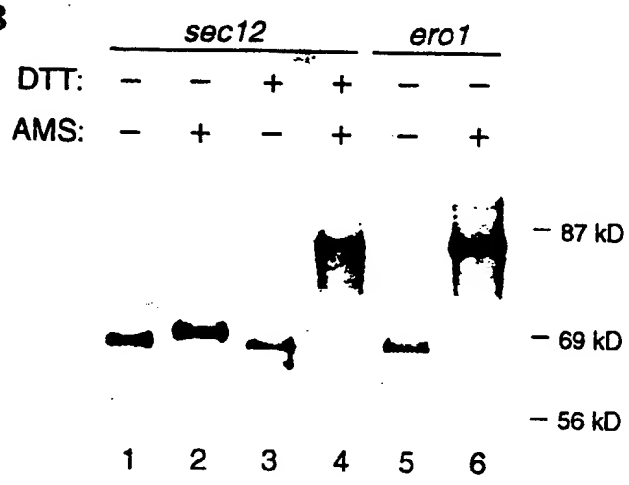

sec12
ero1
DTT: − − + + − −
AMS: − + − + − +
87 kD
69 kD
56 kD
1 2 3 4 5 6

Fig.4 A

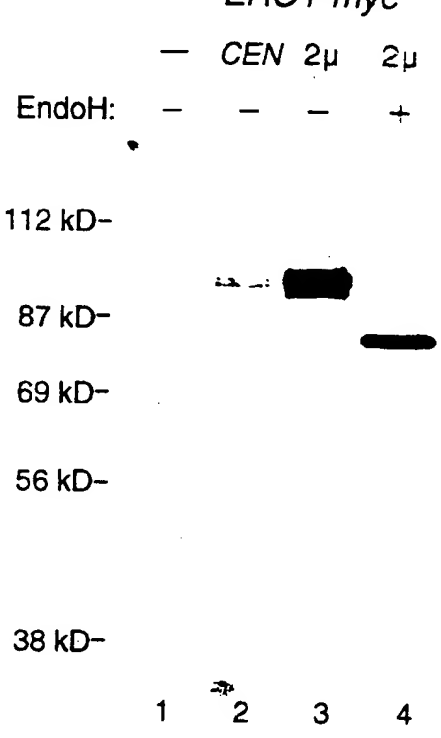

ERO1-myc
— CEN 2μ 2μ
EndoH: – – – +
112 kD–
87 kD–
69 kD–
56 kD–
38 kD–
1 2 3 4

Fig.4 B

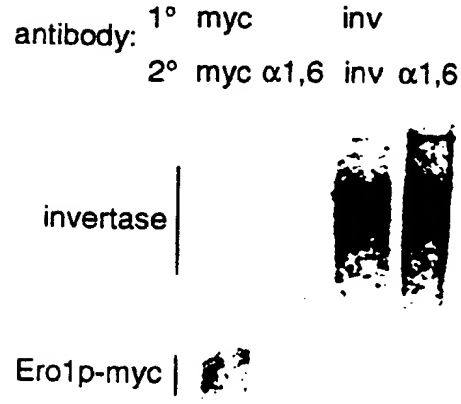

antibody: 1° myc inv
2° myc α1,6 inv α1,6
invertase
Ero1p-myc
1 2 3 4

Fig.5 A

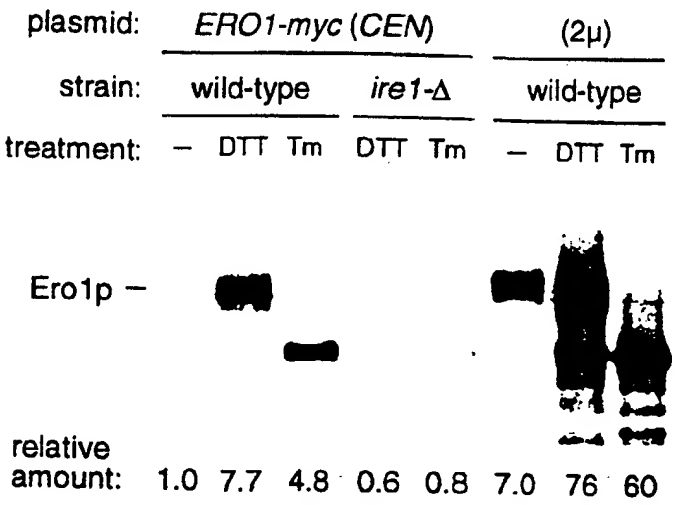

plasmid:
ERO1-myc (CEN)
(2μ)
strain:
wild-type
ire1-Δ
wild-type
treatment:
– DTT Tm DTT Tm – DTT Tm
Ero1p –
relative amount:
1.0 7.7 4.8 0.6 0.8 7.0 76 60

Fig.5 B

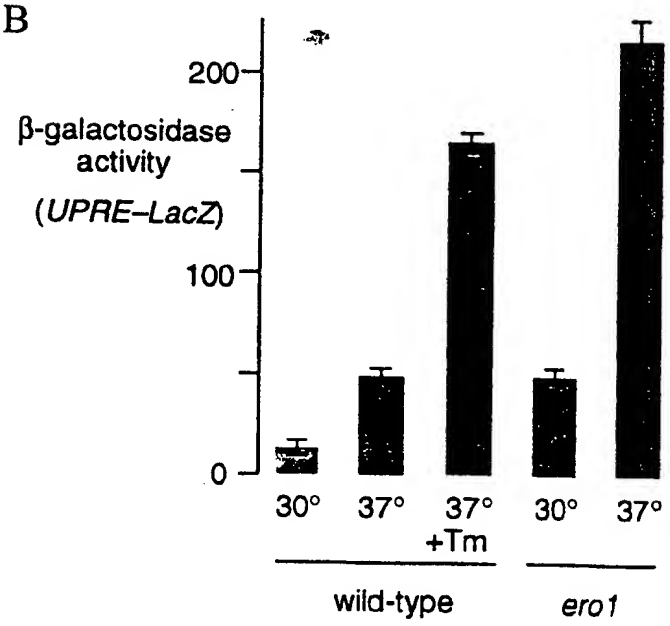

200
β-galactosidase activity
(UPRE–LacZ)
100
0
30°
37°
37° +Tm
30°
37°
wild-type
ero1

Fig.6 A

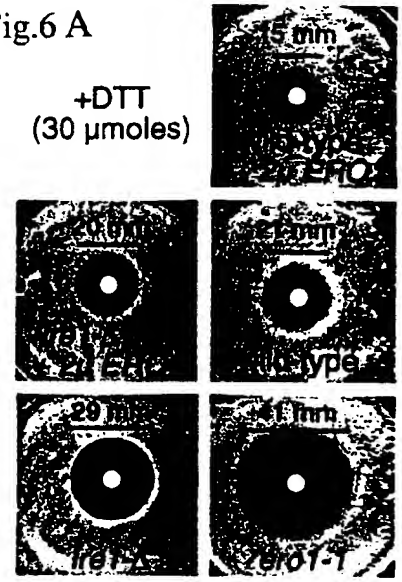

+DTT
(30 µmoles)
5 mm
wild-type
+2µ ERO1
29 mm
ire1-Δ
ero1-1

Fig.6 B

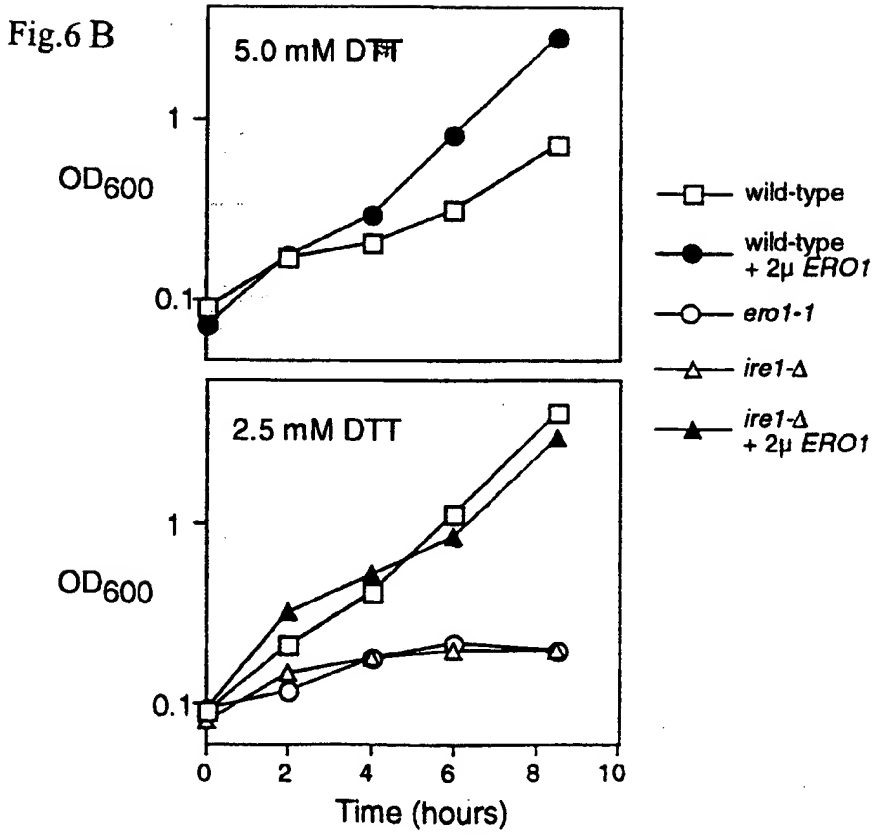

5.0 mM DTT
2.5 mM DTT
OD600
1
0.1
0
2
4
6
8
10
Time (hours)
wild-type
wild-type
+ 2µ ERO1
ero1-1
ire1-Δ
ire1-Δ
+ 2µ ERO1

Fig.6 C

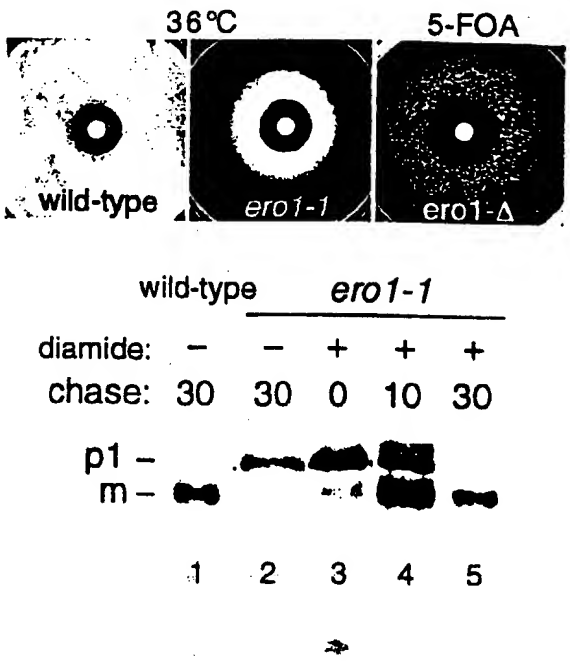

+diamide (6 μmoles)
36°C
5-FOA
wild-type
ero1-1
ero1-Δ
wild-type
ero1-1
diamide: – – + + +
chase: 30 30 0 10 30
p1 –
m –
1 2 3 4 5

Fig.6 D

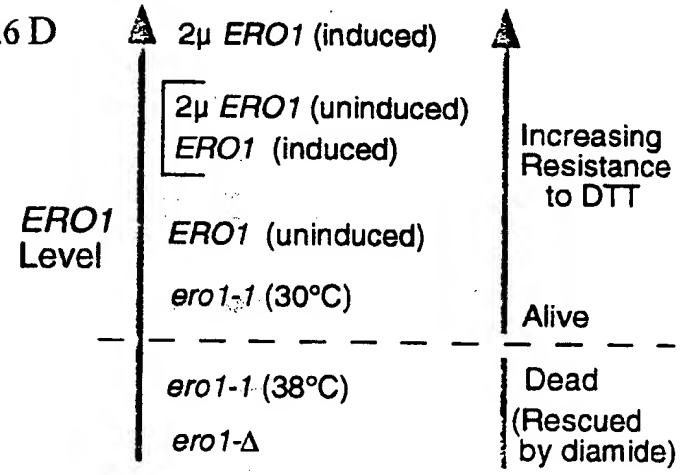

2μ ERO1 (induced)
2μ ERO1 (uninduced)
ERO1 (induced)
ERO1 (uninduced)
ero1-1 (30°C)
ero1-1 (38°C)
ero1-Δ
ERO1 Level
Increasing Resistance to DTT
Alive
Dead (Rescued by diamide)

Fig.7 A

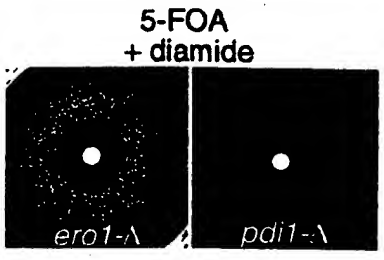


Fig.7 B

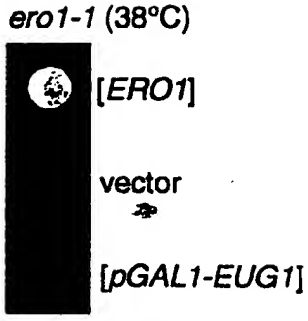
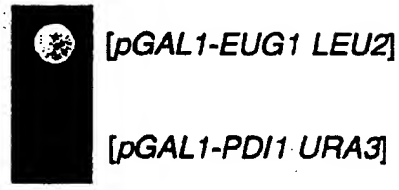


Fig.7 C



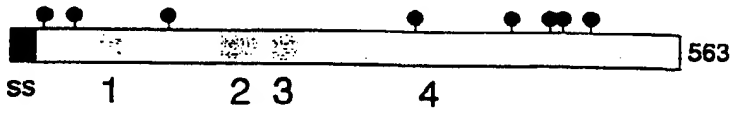


Fig.8 A

1	Sc(73)	LLKSDFFKYFRLDLYKQCSFW	(SEQ ID NO: 12)
	Tb	ITSHPYFRYFKVNLDRECRYW	(SEQ ID NO: 13)
	Dm	LLVKNFFRFYKVNLRQECPFW	(SEQ ID NO: 14)
	Hs	LLESVYFRYYKVNLKRPCPIW	(SEQ ID NO: 15)
2	Sc(176)	AVLIDLTANPERFTGYGGKQAGQIWSTIYQDNC	(SEQ ID NO: 16)
	Tb	ATYVDLLQNPEANTGYSGPKAARVWQAVY DNC	(SEQ ID NO: 17)
	Hs	AEYVDLLLNPERYTGYKGPDAWKIWNVIYEENC	(SEQ ID NO: 18)
3	Sc(219)	AKDAFYRLVSGFHASIGTHLS	(SEQ ID NO: 19)
	Tb	EKALLRQLLSGLHTSITMHVA	(SEQ ID NO: 20)
	Bm	EKRVFYRLISGLHSAITISIA	(SEQ ID NO: 21)
	Hs	EKRAFYRLISGLHASINVHLS	(SEQ ID NO: 22)
4	Sc(332)	LKDEFRSRFKNVTKIMDCVQCDRCRLWGKIQTTGYATALKILF	(SEQ ID NO: 23)
	Sp	FKDSFRKHFRDISRIMDCVGCDKCRLWGKVQITGYGTALKLLL	(SEQ ID NO: 24)
	Tb	LVRQMKRVVHNVTTLMDCVTCEKCRAWGKLETAALATALKIVF	(SEQ ID NO: 25)
	Hs	LKEDFRLHFRNISRIMDCVGCFKCRLWGKLQTQGLGTALKILF	(SEQ ID NO: 26)
	At	LKQHLEKQFRNISAIMDCVGCEKCRLWGKLQILGLGTAL-ILF	(SEQ ID NO: 27)
		* * *	

Fig.8 B

Yeast Strains

Strain	Genotype	Source
CKY8	*MATα ura3-52 leu2-3,112*	Kaiser Lab Collection
CKY10	*MATa ura3-52 leu2-3,112*	"
CKY39	*MATα sec12-4 ura3-52 his4-619*	"
CKY406	*MATα suc2-Δ9 ura3-52 leu2-3,112*	"
CKY560	*MATα sec6-4 ura3-52 leu2-3,112*	This Study
CKY558	*MATa ero1-1 ura3-52 leu2-3,112 ade2*	"
CKY559	*MATα ero1-1 ura3-52 leu2-3,112*	"
CKY561	*MATa ire1-Δ::URA3 ura3-52 leu2-3,112*	"
CKY562	*MATa/α ero1-Δ::LEU2/ERO1 leu2-3,112/leu2-3,112 ura3-52/ura3-52*	"
CKY563	*MATα ero1-Δ::LEU2 ura3-52 leu2-3,112* [pAF82]	"
CKY222	*MATa kar2-159 ura3-52 leu2-3,112*	Mark Rose (MS174)
CKY229	*MATa kar2-203 ura3-52 leu2-3,112 ade2-101*	Mark Rose (MS1032)
CKY190	*MATα KAR2-ΔHDEL suc2-Δ9 ura3-52 leu2-3,112 his4-619*	Mark Rose
CKY395	*MATα pdi1-Δ::TRP1-PDI1-ΔHDEL leu2-3,112::LEU2-UPRE-lacZ ura3-1 his3-11,15 trp1-1 ade2-1 can1-100*	Caroline Shamu (CS297)
CKY564	*MATα pdi1-Δ::HIS3 ura3-1 leu2-3, 112 his3-11,15 trp1-1 ade2-1 can1-100* [pCT37]	Tom Stevens
CKY565	*MATa/α gsh1-Δ1::URA3/gsh1-Δ1::URA3 leu2-Δ1/LEU2 ura3-52/ura3-52 ade2-101/ade2-101 lys2-801/LYS2 trp1-Δ1/TRP1trp5/TRP5*	Martin Grey (M65312)

Fig.9

Consensus secuence for cDNA of mammalian ERO1 (human and mouse)

```
   1 CGCCGCTGGGGCCGGCCCGCACGGCTTCATCTGAGGGCGCACGGCCCGCGACCGAGCGTGCGGACTGGCCTCCCAAGCGT 80

  81 GGGGCGACAAGCTGCCCGAGCTGCA ATG GGC CGC GGC TGG GGA TTC TTG TTT GGA CTC CTG GGC     144
   1                          M   G   R   G   W   G   F   L   F   G   L   L   G      13

 145 GCC GTG TGG CTG CTG CAG TCG GGC CAC GGC GAG GAG CAG CGC CCG GAG ACA GCG GCA CAG  204
  14 A   V   W   L   L   Q   S   G   H   G   E   E   Q   R   P   E   T   A   A   Q    33

 205 CGG TGC TTC TGC CAG GTT AGT GGT TAC CTG GAT GAC TGT ACC TGT GAT GTT GAA ACC ATC  264
  34 R   C   F   C   Q   V   S   G   Y   L   D   D   C   T   C   D   V   E   T   I    53

 265 GAT AGA TTT AAT AAC TAC AGG CTT TTC CCA AGA CTA CAA AAA CTT CTT GAA AGT GAC TAC  324
  54 D   R   F   N   N   Y   R   L   F   P   R   L   Q   K   L   L   E   S   D   Y    73

 325 TTT AGG TAT TAC AAG GTA AAC CTG AAG AGG CCG TGT CCT ATC TGG AAT GAC ATC AGC CAG  384
  74 F   R   Y   Y   K   V   N   L   K   R   P   C   P   I   W   N   D   I   S   Q    93

 385 TGT GGA AGA AGG GAC TGT GCT GTC AAA CCA TGT CAA TCT GAT GAA GTT CCT GAT GGA ATT  444
  94 C   G   R   R   D   C   A   V   K   P   C   Q   S   D   E   V   P   D   G   I   113

 445 AAA TCT GCG AGC TAC AAG TAT TCT GAA GAA GCC AAT AAT CTC ATT GAA GAA TGT GAA CAA  504
 114 K   S   A   S   Y   K   Y   S   E   E   A   N   N   L   I   E   E   C   E   Q   133

 505 GCT GAA CGA CTT GGA GCA GTG GAT GAA TCT CTG AGT GAG GAA ACA CAG AAG GCT GTT CTT  564
 134 A   E   R   L   G   A   V   D   E   S   L   S   E   E   T   Q   K   A   V   L   153

 565 CAG TGG ACC AAG CAT GAT GAT TCT TCA GAT AAC TTC TGT GAA GCT GAT GAT GAC ATT CAG  624
 154 Q   W   T   K   H   D   D   S   S   D   N   F   C   E   A   D   D   D   I   Q   173

 625 TCC CCT GAA GCT GAA TAT GTA GAT TTG CTT CTT AAT CCT GAG CGC TAC ACT GGT TAC AAG  684
 174 S   P   E   A   E   Y   V   D   L   L   L   N   P   E   R   Y   T   G   Y   K   193

 685 GGA CCA GAT GCT TGG AAA ATA TGG AAT GTC ATC TAC GAA GAA AAC TGT TTT AAG CCA CAG  744
 194 G   P   D   A   W   K   I   W   N   V   I   Y   E   E   N   C   F   K   P   Q   213

 745 ACA ATT AAA AGA CCT TTA AAT CCT TTG GCT TCT GGT CAA GGG ACA AGT GAA GAG AAC ACT  804
 214 T   I   K   R   P   L   N   P   L   A   S   G   Q   G   T   S   E   E   N   T   233

 805 TTT TAC AGT TGG CTA GAA GGT CTC TGT GTA GAA AAA AGA GCA TTC TAC AGA CTT ATA TCT  864
 234 F   Y   S   W   L   E   G   L   C   V   E   K   R   A   F   Y   R   L   I   S   253

 865 GGC CTA CAT GCA AGC ATT AAT GTG CAT TTG AGT GCA AGA TAT CTT TTA CAA GAG ACC TGG  924
 254 G   L   H   A   S   I   N   V   H   L   S   A   R   Y   L   L   Q   E   T   W   273

 925 CTG GAA AAG AAA TGG GGT CAC AAT GTC ACA GAG TTC CAG CAG CGC TTT GAT GGG ATT CTG  984
 274 L   E   K   K   W   G   H   N   V   T   E   F   Q   Q   R   F   D   G   I   L   293

 985 ACT GAA GGA GAA GGC CCA CGA AGG CTG AGG AAC TTG TAC TTC CTG TAC CTG ATA GAG TTA 1044
 294 T   E   G   E   G   P   R   R   L   R   N   L   Y   F   L   Y   L   I   E   L   313

1045 AGG GCT CTC TCC AAA GTG CTT CCA TTT TTT GAG CGT CCA GAT TTT CAG CTC TTC ACT GGG 1104
 314 R   A   L   S   K   V   L   P   F   F   E   R   P   D   F   Q   L   F   T   G   333

1105 AAT AAA ATT CAG GAT GAG GAA AAC AAA ATG TTA CTT CTG GAA ATA CTT CAT GAA ATC AAG 1164
 334 N   K   I   Q   D   E   E   N   K   M   L   L   L   E   I   L   H   E   I   K   353

1165 TCA TTT CCT TTG CAT TTT GAT GAG AAT TCA TTT TTT GCT GGG GAT AAA AAA GAA GCA CAC 1224
 354 S   F   P   L   H   F   D   E   N   S   F   F   A   G   D   K   K   E   A   H   373

1225 AAA CTA AAG GAG GAC TTT CGA CTG CAT TTT AGA AAT ATT TCA AGA ATT ATG GAT TGT GTT 1284
 374 K   L   K   E   D   F   R   L   H   F   R   N   I   S   R   I   M   D   C   V   393

1285 GGT TGT TTT AAA TGT CGT CTG TGG GGA AAG CTT CAG ACT CAG GGT TTG GGC ACT GCT CTG 1344
 394 G   C   F   K   C   R   L   W   G   K   L   Q   T   Q   G   L   G   T   A   L   413

1345 AAG ATC TTA TTT TCT GAG AAA TTG ATA GCA AAT ATG CCA GAA AGT GGA CCT AGT TAT GAA 1404
 414 K   I   L   F   S   E   K   L   I   A   N   M   P   E   S   G   P   S   Y   E   433

1405 TTC CAT CTA ACC AGA CAA GAA ATA GTA TCA TTA TTC AAC GCA TTT GGA AGA ATT TCT ACA 1464
 434 F   H   L   T   R   Q   E   I   V   S   L   F   N   A   F   G   R   I   S   T   453

1465 AGT GTG AAA GAA TTA GAA AAC TTC AGG AAC TTG TTA CAG AAT ATT CAT TAA AGAAAACAAGCT 1527
 454 S   V   K   E   L   E   N   F   R   N   L   L   Q   N   I   H   *               470

1528 GATATGTGCCTGTTTCTGGACAATGGAGGCGAAAGAGTGGAATTTCATTCAAAGGCATAATAGCAATGACAGTCTTAAGC 1607

1608 CAAACATTTTATATAAAGTTGCTTTTGTAAAGGAGAATTATATTGTTTTAAGTAAACACATTTTTAAAAATTGTGTTAAG 1687

1688 TCTATGTATAATACTACTGTGAGTAAAAGTAATACTTTAATAATGTGGTACAAATTTTAAAGTTTAATATTGAATAAAAG 1767

1768 GAGGATTATCAAATTCATATATGATAAAAGTGAATGTTCTAAGTCTCTCAAACTAGCGGTTTATGTAATAATATGTAATA 1847

1848 TAAA   1851
```

Figure 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/16593

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 68.1, 233, 252.3, 254.11, 320.1, 325, 410; 530/387.9; 536/23.2; 935/22

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE (STN), MEDLINE, REGISTRY, EMBASE, BIOSIS
search terms: DSBA, disulfide oxidoreductase, dsbA, dsb, dsbC,disulfide, author/inventor name searches, ERO1, SEC81

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NG et al. Cloning and expression of the gene for a protein disulfide oxidoreductase from Azotobacter vinelandii: complementation of an Escherichia coli dsbA mutant strain. Gene. October 1997. Vol. 188. No. 1. pages 109-113, (see entire document).	1-36
A	RUDDOCK et al. pH-dependence of the dithiol-oxidizing activity of DsbA (a periplasmic protein thiol:disulfide oxidoreductase) and protein disulfide-isomerase: studies with a novel simple peptide substrate. Biochemical Journal. May 1996. Vol. 315. No. 3. pages 1001-1005, see entire document.	1-36
A	US 5,639,635 A (JOLY et al.) 17 June 1997, entire document.	1-36

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 29 SEPTEMBER 1998	Date of mailing of the international search report 26 OCT 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer EINAR STOLE Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/16593

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	US 5,789,199 A (JOLY et al.) 04 August 1998, see entire document.	1-36

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/16593

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07H 21/04; C07K 16/00; C12N 1/14, 1/20, 5/00, 9/90, 15/00; C12P 21/06; C12Q 1/68

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 68.1, 233, 252.3, 254.11, 320.1, 325, 410; 530/387.9; 536/23.2; 935/22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/16593

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07H 21/04; C07K 16/00; C12N 1/14, 1/20, 5/00, 9/90, 15/00; C12P 21/06; C12Q 1/68

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 68.1, 233, 252.3, 254.11, 320.1, 325, 410; 530/387.9; 536/23.2; 935/22